

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

Consensus guidelines for the detection of immunogenic cell death

This is a pre print version of the following article:

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/152813> since

Published version:

DOI:10.4161/21624011.2014.955691

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)



UNIVERSITÀ DEGLI STUDI DI TORINO

This is an author version of the contribution published on:

Questa è la versione dell'autore dell'opera:

[Oncoimmunology, 3, 9:e955691, 2014, doi: 10.4161/21624011.2014.955691]

The definitive version is available at:

La versione definitiva è disponibile alla URL:

[<http://www.tandfonline.com/loi/koni20>]

Consensus guidelines for the detection of immunogenic cell death

Oliver Kepp^{1-3,*}, Laura Senovilla^{2-4,*}, Ilio Vitale⁵, Erika Vacchelli^{1,2,6}, Sandy Adjemian^{1,2,6,7}, Patrizia Agostinis⁸, Lionel Apetoh⁹⁻¹¹, Fernando Aranda^{1,2,6}, Vincenzo Barnaba^{12,13}, Norma Bloy^{1,2,6}, Laura Bracci¹⁴, Karine Breckpot¹⁵, David Brough¹⁶, Aitziber Buqué^{1,2,6}, Maria G. Castro¹⁷, Mara Cirone¹⁸, Maria I. Colombo¹⁹, Isabelle Cremer^{2,20,21}, Sandra Demaria²², Luciana Dini²³, Aristides Eliopoulos^{24,25}, Alberto Faggioni¹⁸, Silvia C. Formenti²⁶, Jitka Fučíková^{27,28}, Lucia Gabriele¹⁴, Udo S. Gaipl²⁹, Jérôme Galon^{2,20,30,31}, Abhishek Garg⁸, François Ghiringhelli⁹⁻¹¹, Nathalia A. Giese³², Zong Sheng Guo³³, Akseli Hemminki³⁴, Martin Herrmann³⁵, James W. Hodge³⁶, Stefan Holdenrieder³⁷, Jamie Honeychurch³⁸, Hong-Min Hu^{39,40}, Xing Huang¹⁻³, Tim M. Illidge³⁸, Koji Kono^{41,42}, Mladen Korbelik⁴³, Dmitri V. Krysko⁸, Sherene Loi⁴⁴, Pedro R. Lowenstein¹⁷, Enrico Lugli⁴⁵, Yuting Ma^{1,2,6}, Frank Madeo⁴⁶, Angelo A. Manfredi^{47,48}, Isabelle Martins^{6,49,50}, Polly Matzinger⁵¹, Domenico Mavilio⁴⁵, Laurie Menger^{1,2,6,52}, Nicolò Merendino⁵³, Michael Michaud^{1,2,6}, Gregoire Mignot⁵⁴, Karen L. Mossman^{55,56}, Gabriele Multhoff⁵⁷, Rudolf Oehler⁵⁸, Fabio Palombo^{12,13}, Theodoros Panaretakis⁵⁹, Jonathan Pol^{1,2,6}, Enrico Proietti¹⁴, Jean-Ehrland Ricci⁶⁰⁻⁶³, Chiara Riganti⁶⁴, Patrizia Rovere-Querini^{47,48}, Anna Rubartelli⁶⁵, Antonella Sistigu⁵, Mark J. Smyth^{66,67}, Juergen Sonnemann⁶⁸, Radek Spisek^{27,28}, John Stagg⁶⁹, Abdul Q. Sukkurwala^{1,2,6,70}, Eric Tartour^{71,72}, Andrew Thorburn⁷³, Stephen H. Thorne³³, Peter Vandenabeele⁷⁴⁻⁷⁶, Francesca Velotti⁵³, Samuel T. Workenhe^{55,56}, Haining Yang⁷⁷, Wei-Xing Zong⁷⁸, Laurence Zitvogel^{4,6,79}, Guido Kroemer^{1-3,30,72,**} and Lorenzo Galluzzi^{1,2,6,30,**}

¹Equipe 11 labellisée par la Ligue Nationale contre le Cancer, Centre de Recherche des Cordeliers; Paris, France; ²INSERM, U1138; Paris, France; ³Metabolomics and Cell Biology Platforms, Gustave Roussy; Villejuif, France; ⁴INSERM, U1015; Villejuif, France; ⁵Regina Elena National Cancer Institute; Rome, Italy; ⁶Gustave Roussy Cancer Campus; Villejuif, France; ⁷Molecular Cell Biology Laboratory, Department of Immunology, Institute of Biomedical Sciences, University of São Paulo; São Paulo, Brazil; ⁸Cell Death Research and Therapy (CDRT) Laboratory, Department of Cellular and Molecular Medicine, University of Leuven; Leuven, Belgium; ⁹INSERM, UMR866; Dijon, France; ¹⁰Centre Georges François Leclerc; Dijon, France; ¹¹Université de Bourgogne; Dijon, France; ¹²Dipartimento di Internal Medicine and Medical Sciences, University of Rome La Sapienza; Rome, Italy; ¹³Istituto Pasteur, Fondazione Cenci Bolognetti; Rome, Italy; ¹⁴Department of Hematology,

Oncology and Molecular Medicine, Istituto Superiore di Sanità (ISS); Rome, Italy;¹⁵Laboratory of Molecular and Cellular Therapy (LMCT), Department of Biomedical Sciences Medical School of the Free University of Brussels (VUB); Jette, Belgium;¹⁶Faculty of Life Sciences, University of Manchester; Manchester, UK;¹⁷Department of Neurosurgery and Cell and Developmental Biology, University of Michigan School of Medicine; Ann Arbor, MI, USA;¹⁸Department of Experimental Medicine, University of Rome La Sapienza; Rome, Italy;¹⁹Laboratorio de Biología Celular y Molecular, Instituto de Histología y Embriología (IHEM), Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, CONICET; Mendoza, Argentina;²⁰Université Pierre et Marie Curie/Paris VI; Paris, France;²¹Equipe 13, Centre de Recherche des Cordeliers; Paris, France;²²Department of Pathology, New York University School of Medicine; New York, NY, USA;²³Department of Biological and Environmental Science and Technology (DiSTeBA), University of Salento; Lecce, Italy;²⁴Molecular and Cellular Biology Laboratory, Division of Basic Sciences, University of Crete Medical School; Heraklion, Greece;²⁵Institute of Molecular Biology and Biotechnology, Foundation of Research and Technology - Hellas; Heraklion, Greece;²⁶Department of Radiation Oncology, New York University School of Medicine and Langone Medical Center, New York, NY, USA;²⁷Department of Immunology, 2nd Faculty of Medicine and University Hospital Motol, Charles University; Prague, Czech Republic;²⁸Sotio; Prague, Czech Republic;²⁹Department of Radiation Oncology, University Hospital Erlangen, University of Erlangen-Nürnberg; Erlangen, Germany;³⁰Université Paris Descartes/Paris V; Sorbonne Paris Cité; Paris, France;³¹Laboratory of Integrative Cancer Immunology; Centre de Recherche des Cordeliers; Paris, France;³²European Pancreas Centre, Department of Surgery, University Hospital Heidelberg; Heidelberg, Germany;³³Department of Surgery, University of Pittsburgh; Pittsburgh, PA, USA;³⁴Cancer Gene Therapy Group, Transplantation laboratory, Haartman Institute, University of Helsinki; Helsinki, Finland;³⁵Department of Internal Medicine 3, University of Erlangen-Nuremberg; Erlangen, Germany;³⁶Laboratory of Tumor Immunology and Biology, Center for Cancer Research, National Cancer Institute (NCI), National Institutes of Health (NIH); Bethesda, MD, USA;³⁷Institute of Clinical Chemistry and Clinical Pharmacology, University Hospital Bonn; Bonn, Germany;³⁸Faculty of Medical and Human Sciences, Institute of Cancer Studies, Manchester Academic Health Sciences Centre, University of Manchester; Manchester, UK;³⁹Cancer Research and Biotherapy Center, Second Affiliated Hospital of Southeast University; Nanjing, China;⁴⁰Laboratory of Cancer Immunobiology, Earle A. Chiles Research Institute, Providence Portland Medical Center; Portland, OR, USA;⁴¹Department of Surgery, National University of Singapore; Singapore, Singapore;⁴²Cancer Science Institute of Singapore, National University of Singapore; Singapore, Singapore;⁴³British Columbia Cancer Agency; Vancouver, Canada;⁴⁴Division of

Cancer Medicine and Division of Research, Peter MacCallum Cancer Centre; East Melbourne, Victoria, Australia; ⁴⁵Unit of Clinical and Experimental Immunology, Humanitas Clinical and Research Center; Milan, Italy; ⁴⁶Institute of Molecular Biosciences, University of Graz; Graz, Austria; ⁴⁷University Vita-Salute San Raffaele; Milano, Italy; ⁴⁸San Raffaele Scientific Institute; Milano, Italy; ⁴⁹INSERM, U1030; Villejuif, France; ⁵⁰Faculté de Médecine, Université Paris-Sud/Paris XI; Kremlin-Bicêtre, France; ⁵¹Ghost Laboratory, Laboratory of Cellular and Molecular Immunology, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH); Bethesda, MD, US; ⁵²Cancer Immunology Unit, Research Department of Haematology, University College London (UCL) Cancer Institute; London, UK; ⁵³Department of Ecological and Biological Sciences (DEB), Tuscia University; Viterbo, Italy; ⁵⁴Cellular and Molecular Immunology and Endocrinology, Oniris; Nantes, France; ⁵⁵Department of Pathology and Molecular Medicine, McMaster Immunology Research Centre; Hamilton, Canada; ⁵⁶Institute for Infectious Disease Research, McMaster University; Hamilton, Canada; ⁵⁷Department of Radiation Oncology, Klinikum rechts der Isar, Technical University of Munich; Munich, Germany; ⁵⁸Comprehensive Cancer Center, Medical University of Vienna; Vienna, Austria; ⁵⁹Department of Oncology-Pathology, Karolinska Institute; Stockholm, Sweden; ⁶⁰INSERM, U1065; Nice, France; ⁶¹Equipe “Contrôle Métabolique des Morts Cellulaires”, Centre Méditerranéen de Médecine Moléculaire (C3M); Nice, France; ⁶²Faculté de Médecine, Université de Nice Sophia Antipolis; Nice, France; ⁶³Centre Hospitalier Universitaire de Nice; Nice, France; ⁶⁴Department of Oncology and Subalpine Center for Research and Experimental Medicine (CeRMS), University of Turin; Turin, Italy; ⁶⁵Cell Biology Unit, Azienda Ospitaliera Universitaria San Martino, Istituto Nazionale per la Ricerca sul Cancro; Genova, Italy; ⁶⁶Immunology in Cancer and Infection Laboratory, QIMR Berghofer Medical Research Institute; Herston, Australia; ⁶⁷School of Medicine, University of Queensland; Herston, Australia; ⁶⁸Department of Paediatric Haematology and Oncology, Jena University Hospital, Children's Clinic; Jena, Germany; ⁶⁹Centre de Recherche du Centre Hospitalier de l'Université de Montréal, Faculté de Pharmacie, Université de Montréal; Montréal, Canada; ⁷⁰Department of Pathology, Dow International Medical College, Dow University of Health Sciences; Karachi, Pakistan; ⁷¹INSERM, U970; Paris, France; ⁷²Pôle de Biologie, Hôpital Européen Georges Pompidou, AP-HP; Paris, France; ⁷³Department of Pharmacology, University of Colorado School of Medicine; Aurora, CO, USA; ⁷⁴VIB Inflammation Research Center; Gent, Belgium; ⁷⁵Department of Biomedical Molecular Biology, Ghent University; Gent, Belgium; ⁷⁶Methusalem Program, Ghent University; Gent, Belgium; ⁷⁷University of Hawaii Cancer Center; Honolulu, HI, USA; ⁷⁸Department of Molecular Genetics and Microbiology, Stony Brook University; Stony

Brook, NY, USA; ⁷⁹Centre d'Investigation Clinique Biothérapie 507 (CICBT507), Gustave Roussy
Cancer Campus, Villejuif, France.

*equally contributed to this work

**share senior co-authorship

Correspondence to: **Dr. Lorenzo Galluzzi or Dr. Guido Kroemer**
INSERM, U848
Gustave Roussy Comprehensive Cancer Center, PR1
39, rue Camille Desmoulins
F-94805 Villejuif
France
Tel. +33 1 4211 4516 or +33 1 4211 6046
Fax +33 1 4211 6047
e-mail: deadoc@vodafone.it or kroemer@orange.fr

Running title: Definition and assessment of ICD

Keywords: ATP release; autophagy; calreticulin, endoplasmic reticulum stress; HMGB1;
immunotherapy.

Conflict of interest: AH owns a share of Oncos Therapeutics Ltd. (Helsinki, Finland) and TILT
Biotherapeutics Ltd. (Helsinki, Finland), and is a legal employee of the latter.

Abbreviations: APC, antigen-presenting cell; ATF6, activating transcription factor 6; BAK1, BCL2-antagonist/killer 1; BAX, BCL2-associated X protein; BCL2, B-cell CLL/lymphoma 2 protein; CALR, calreticulin; CTL, cytotoxic T lymphocyte; $\Delta\psi_m$, mitochondrial transmembrane potential; DAMP, damage-associated molecular pattern; DAPI, 4',6-diamidino-2-phenylindole; DiOC₆(3), 3,3'-dihexyloxacarbocyanine iodide; EIF2A, eukaryotic translation initiation factor 2A; ER, endoplasmic reticulum; FLT3LG, *fms*-related tyrosine kinase 3 ligand; G3BP1, GTPase activating protein (SH3 domain) binding protein 1; GFP, green fluorescent protein; H2B, histone 2B; HMGB1, high mobility group box 1; HSP, heat shock protein; HSV-1, herpes simplex virus type I; ICD, immunogenic cell death; IFN, interferon; IL, interleukin; MOMP, mitochondrial outer membrane permeabilization; PDIA3, protein disulfide isomerase family A, member 3; PI, propidium iodide; RFP, red fluorescence protein; TLR, Toll-like receptor; XBP1, X-box binding protein 1.

Abstract

For long, apoptotic cells have been considered as intrinsically tolerogenic or unable to elicit immune responses specific for dead cell-associated antigens. However, multiple stimuli can trigger a functionally peculiar type of apoptotic demise that does not go unnoticed by the adaptive arm of the immune system, which we named “immunogenic cell death” (ICD). ICD is preceded or accompanied by the emission of a series of immunostimulatory damage-associated molecular patterns (DAMPs) in a precise spatiotemporal configuration. Several anticancer agents that have been successfully employed in the clinic for decades, including various chemotherapeutics and radiotherapy, can elicit ICD. Moreover, defects in the components that underlie the capacity of the immune system to perceive cell death as immunogenic negatively influence disease outcome among cancer patients treated with ICD inducers. Thus, ICD has profound clinical and therapeutic implications. Unfortunately, the gold-standard approach to detect ICD relies on vaccination experiments involving immunocompetent murine models and syngeneic cancer cells, an approach that is incompatible with large screening campaigns. Here, we outline strategies conceived to detect surrogate markers of ICD *in vitro* and to screen large chemical libraries for putative ICD inducers, based on a high-content, high-throughput platform that we recently developed. Such platform allows for the detection of multiple DAMPs, like cell surface-exposed calreticulin, extracellular ATP and high mobility group box 1 (HMGB1), and/or the processes that underlie their emission, such as endoplasmic reticulum stress, autophagy and necrotic plasma membrane permeabilization. We surmise that this technology will facilitate the development of next-generation anticancer regimens, which kill malignant cells and simultaneously convert them into a cancer-specific therapeutic vaccine.

Introduction

Cell death can be classified based on several parameters, including morphological manifestations, biochemical features, kinetic considerations and functional outcomes.¹⁻⁷ This said, how cell death has been investigated and conceived since its pristine descriptions (dating back to the mid-19th century)⁸ has obviously evolved along with the technological advances that have been made throughout the last one and a half centuries.^{9,10} Thus, morphology-based classifications postulating the existence of three cell death subroutines (i.e., type I, type II and type III cell death)^{2,11-14} have been progressively abandoned in favor of definitions that rely on objectively quantifiable functional features.^{3,15-19} Alongside, the long-standing conception according to which distinct types of cell death like apoptosis and necrosis would constitute mutually exclusive and diametrically opposed entities has been refuted. In particular, throughout the past two decades it has become clear that: (1) apoptosis is not the sole type of regulated cell death that contributes to (post-)embryonic development and adult tissue homeostasis;²⁰ (2) similar to apoptosis, necrosis can occur in a regulated fashion, i.e., it can involve a genetically encoded molecular machinery;^{4,5,21} (3) similar to their necrotic counterparts, apoptotic cells can sometimes be detected by the immune system and elicit an adaptive immune response specific for dead cell-associated antigens.^{6,7,22,23} Thus, although apoptosis as a physiological process involved in (post-)embryonic development and tissue homeostasis invariably fails to engage the adaptive branch of the immune system,^{24,25} specific stimuli can promote an immunogenic variant of regulated cell death that manifests with both morphological and biochemical features of apoptosis.^{2,3,6} Of note, defects in the clearance of apoptotic cells by professional phagocytes have been associated with autoimmune conditions such as systemic lupus erythematosus (SLE) and chronic inflammation.^{26,27} However, it remains unclear whether this reflects the immunogenic potential of intact apoptotic corpses or the insurgence of secondary necrosis.

Back in 2005, we were the first to report the unexpected finding that murine colorectal carcinoma

CT26 cells as well as murine fibrosarcoma MCA205 cells exposed to a lethal dose of doxorubicin *in vitro* are capable of vaccinating syngeneic mice against a subsequent challenge with living cells of the same type.²² We dubbed such a functionally peculiar variant of cellular demise, manifesting with an apoptotic morphology and depending on the activity of apoptotic caspases, “immunogenic cell death” (ICD).²² It turned out that the unsuspected ability of doxorubicin (an anthracycline employed for the treatment of various carcinomas) to trigger ICD as a standalone intervention, hence converting dying cancer cells into a vaccine that is efficient in the absence of adjuvants, is shared by a relatively restricted set of lethal triggers.²⁸⁻³³ These include, but perhaps are not limited to, mitoxantrone and epirubicin (two other anthracyclines currently used in the clinic),³⁴⁻³⁷ bleomycin (a glycopeptide antibiotic endowed with antineoplastic properties),³⁸ oxaliplatin (a platinum derivative generally employed against colorectal carcinoma),³⁹⁻⁴² cyclophosphamide (an alkylating agent approved for the treatment of neoplastic and autoimmune conditions),⁴³⁻⁴⁸ etoposide (a topoisomerase inhibitor currently used for the treatment of several neoplasms) combined with the chemical inhibitor of glycolysis 2-deoxyglucose,^{49,50} patupilone (a microtubular inhibitor that has not yet been approved for use in humans),⁵¹⁻⁵³ septacidin (an antifungal antibiotic produced by *Streptomyces fibriatus*)^{54,55} specific forms of radiation therapy,^{34,56-64} photodynamic therapy (a clinically approved anticancer intervention that involves the administration of a photosensitizing agent followed by light irradiation),⁶⁵⁻⁷³ high hydrostatic pressure,⁷⁴ multiple oncolytic viruses,⁷⁵⁻⁸³ replication-defective viral vectors encoding a potentially cytotoxic product (e.g., thymidine kinase from herpes simplex virus type I, HSV-1) combined with viruses expressing an immunostimulatory molecule (e.g., *fms*-related tyrosine kinase 3 ligand, FLT3LG),⁸⁴ the clinically employed proteasomal inhibitor bortezomib,⁸⁵⁻⁸⁷ shikonin (a component of Chinese herbal medicine),⁸⁸ a monoclonal antibody specific for the epidermal growth factor receptor (EGFR),⁸⁹ capsaicin (a neurotoxic derivative of homovanillic acid found in chili peppers),^{90,91} and perhaps the n3-polyunsaturated fatty acid docosahexaenoic acid,⁹² as well as the transgene-driven expression of SMAC mimetics.^{93,94} In addition, some interventions are capable of converting non-immunogenic

instances of cell death into *bona fide* ICD. These maneuvers include the administration of cardiac glycosides, which are particularly powerful in this respect as they promote *per se* all major manifestations of ICD (see below),⁹⁵⁻⁹⁷ or zoledronic acid (a bisphosphonate currently approved to treat osteoporosis and to prevent skeletal fractures in cancer patients with bone metastases),^{98,99} as well as the provision of co-stimulatory signals via CD40.¹⁰⁰ This said, it should be kept in mind that the capacity of a given agent to cause ICD or exacerbate the immunogenicity of apoptosis cannot be predicted yet from its structural or chemical properties, since molecules as similar to each other as oxaliplatin and cisplatin do not share this functional profile.^{39,40}

The notion that apoptotic cancer cells do not always go undetected by the immune system has profound clinical repercussions.¹⁰¹ First, it implies that the immune system, at least under specific circumstances, can mount an adaptive immune response against (self) malignant cells, hence mediating antineoplastic effects or contributing to the therapeutic activity of conventional anticancer regimens. This concept represents the theoretical foundation of modern tumor immunology and anticancer immunotherapy.^{22,102,103} As a matter of fact, many chemotherapeutics that have been successfully used in the clinic throughout the past century have recently been discovered to mediate off-target immunostimulatory effects, ICD being one of the underlying mechanisms (though not the sole).¹⁰⁴⁻¹⁰⁶ Second, it implies that a large number of parameters reflecting the immunological competence of the host, including the type, quantity and localization of tumor-infiltrating lymphoid and myeloid cells,¹⁰⁷⁻¹¹³ the amount of blood-borne memory T cells that are able to recognize antigens associated with apoptotic cancer cells,¹¹⁴ the circulating levels of various ICD-associated biomarkers, including the non-histone chromatin-binding protein high mobility group box 1 (HMGB1),^{46,115-117} as well as genetic polymorphisms affecting virtually all facets of the immune response,^{41,108,118,119} may be endowed with a robust prognostic or predictive value. This notion has already been demonstrated in several ICD-related clinical scenarios. Thus, the relative abundance of tumor-infiltrating CD8⁺ cytotoxic T lymphocytes (CTLs) and

CD4⁺CD25⁺FOXP3⁺ regulatory T cells reportedly predicts the propensity of breast carcinoma patients to benefit from anthracycline- or oxaliplatin-based chemotherapy, respectively.^{52,120} Along similar lines, single nucleotide polymorphisms in the genes coding for ICD-relevant receptors such as Toll-like receptor 4 (TLR4) and purinergic receptor P2X, ligand-gated ion channel, 7 (P2RX7) have been shown to influence disease outcome among breast carcinoma patients treated with anthracycline-based chemotherapy.^{41,119} Taken together, these observations demonstrate that the induction of ICD is a therapeutically relevant objective, calling for the identification of novel ICD inducers and molecules that improve the immunogenicity of conventional variants of apoptosis.

After summarizing the main molecular and cellular determinants that underlie ICD, we discuss the assays that are currently available for the detection of surrogate ICD markers and how these methods can be combined into a platform that is compatible with high-content, high-throughput applications. We surmise that this methodological approach will accelerate the discovery and development of therapeutic regimens that kill malignant cells in an immunogenic fashion.

Immunogenic cell death signaling

According to current models, ICD relies on the ability of specific stimuli to kill cells while provoking the spatiotemporally coordinated emission of immunogenic signals.^{7,121-129} Such signals are conveyed by damage-associated molecular patterns (DAMPs), i.e., molecules that are not accessible by the immune system in physiological conditions but are released or exposed on the outer leaflet of the plasma membrane during cytoprotective stress responses or upon cell death.^{103,130-133} Similar to their microbial counterparts, many (but not all) DAMPs exert robust immunostimulatory effects upon binding to pattern recognition receptors (PRRs) expressed by immune cells.¹²¹ So far, three DAMPs have been attributed a key role in the immunogenic potential of virtually all ICD inducers: the endoplasmic reticulum (ER) chaperone calreticulin (CALR),^{34,65,126,134-136} ATP,^{66,124,137-143} and HMGB1.^{41,46,115,116,144-147} In addition, many DAMPs have been shown to contribute to the immunogenicity of cell death in a limited amount of experimental scenarios. These include immunostimulatory cytokines like interferon α (IFN α),^{148,149} various chaperones of the heat-shock protein (HSP) family, notably heat shock 70kDa protein 1A (HSPA1A, best known as HSP70) and heat shock protein 90kDa alpha (cytosolic), class A member 1 (HSP90AA1, best known as HSP90),^{65,71,85,90,145,150-153} sphingomyelin metabolites (e.g., ceramide and sphingosine-1-phosphate),¹⁵⁴ a plethora of mitochondrial products (e.g., mitochondrial DNA, *N*-formylated peptides, cardiolipin),¹⁵⁵⁻¹⁵⁷ cytosolic components like urate and F-actin,¹⁵⁸⁻¹⁶¹ as well as products of the breakdown of the extracellular matrix (e.g., hyaluronan fragments).^{162,163}

CALR gets exposed on the cell surface early in the course of ICD, i.e., before the apoptosis-associated shuffling of phosphatidylserine between the inner and outer leaflet of the plasma membrane.^{34,164,165} The molecular mechanisms underlying this ICD hallmark have been dissected in detail and appear to involve three distinct signaling modules: (1) an ER stress module centered around the phosphorylation of eukaryotic translation initiation factor 2A (EIF2A) and the resultant arrest in protein synthesis; (2) an apoptotic module involving the activation of caspase-8 and the

consequent cleavage of B-cell receptor-associated protein 31 (BCAP31) as well as the pro-apoptotic Bcl-2 family members BCL2-associated X protein (BAX) and BCL2-antagonist/killer 1 (BAK1); and (3) an exocytosis module requiring the actin cytoskeleton as well as vesicle-associated membrane protein 1 (VAMP1) and synaptosomal-associated protein, 25kDa (SNAP25), two proteins involved in intracellular vesicular trafficking.³⁶ Moreover, in some (but not all) models of ICD,⁶⁷ CALR obligatorily translocates to the cell surface together with another ER chaperone, protein disulfide isomerase family A, member 3 (PDIA3).^{36,37} Upon binding to low density lipoprotein receptor-related protein 1 (LRP1, also known as CD91), membrane-exposed CALR delivers a major phagocytic signal to professional antigen-presenting cells (APCs) such as dendritic cells, de facto improving their capacity to take up dead cells and their corpses.^{66,91,166-173} Interestingly, the phagocytosis-stimulatory effects of CALR is robustly counterbalanced by CD47, which is highly expressed by a large panel of solid and hematopoietic tumors.¹⁶⁶ This latter observation suggests that various neoplasms benefit from avoiding the effects of CALR exposure, perhaps as this prevents the elicitation of an adaptive immune response against the malignant cells that “physiologically” succumb in the course of oncogenesis and tumor progression. Alternatively, the phagocytosis-inhibitory activity of CD47 may confer tumors with an advantage by increasing the local availability of macromolecules derived from the spontaneous demise (and degradation) of some of their cellular constituents. This possibility has not yet experimentally addressed.

The ICD-associated release of ATP proceeds through a complex mechanism that involves (1) the apparent relocation of vesicular ATP stores from lysosomes to autolysosomes; (2) the redistribution of lysosomal-associated membrane protein 1 (LAMP1) to the plasma membrane; (3) Rho-associated, coiled-coil containing protein kinase 1 (ROCK1)-mediated, myosin II-dependent cellular blebbing; and (4) the opening of pannexin 1 (PANX1) channels, 4 processes that rely on caspases.^{140,142,174} In a vast majority of models, the secretion of ATP by cells exposed to ICD inducers requires an intact autophagic machinery.^{83,138,139,175} In these settings, the genetic or

pharmacological inhibition of autophagy limits ATP release by cells succumbing to ICD and hence negatively influences their ability to elicit an adaptive immune response upon inoculation in immunocompetent syngeneic mice.^{60,138,139} Along similar lines, the chemical inducer of autophagy STF-62247 increases the immunostimulatory potential of ICD as triggered by chlorin-e6-based photodynamic therapy (MK, unpublished observations). However, this does not seem to apply to all ICD inducers.⁶⁸ Thus, the ability of hypericin-based photodynamic therapy to induce the secretion of ATP does not appear to change in autophagy-deficient versus autophagy-proficient cells.^{68,70,176} Moreover, the former respond to hypericin-based photodynamic therapy by exposing higher amounts of CALR on the plasma membrane than the latter, hence exhibiting a superior immunogenic potential.^{68,70,176} Possibly, this reflects the incapacity of autophagy-deficient cells to clear oxidized proteins, resulting in an aggravation of the ER stress response that underlies CALR exposure in the course of ICD.^{68,70,176} Irrespective of these variations, extracellular ATP operates as a strong chemoattractant and promotes not only the recruitment of immune cells to sites of ICD, but also their differentiation, an effect that depends on purinergic receptor P2Y₂, G-protein coupled, 2 (P2RY2).^{141,177-179} Moreover, extracellular ATP promotes the activation of the NLR family, pyrin domain containing 3 (NLRP3) inflammasome in APCs, hence stimulating the processing and release of interleukin (IL)-1 β and IL-18.^{119,180-189} In line with this notion, the immunogenic potential of cells succumbing to ICD can be significantly reduced by pharmacological or genetic interventions that limit the availability of ATP in the pericellular space, such as the administration of recombinant apyrase (an ATP-degrading enzyme) or the transfection-enforced overexpression of ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1, best known as CD39), which converts ATP into ADP and AMP.¹⁹⁰ Intriguingly, CD39 and 5'-nucleotidase, ecto (NT5E, best known as CD73), which transforms AMP into adenosine, are often overexpressed by malignant tissues. This reflects the advantage conferred to cancer cells by the conversion of extracellular ATP, which promotes immunosurveillance, into adenosine, which exerts potent immunosuppressive effects.¹⁹¹⁻¹⁹⁷ Of note, autophagy is also important for the perception of cell death as immunogenic

because it contributes to several aspects of cellular immune responses, including the differentiation, survival and activation of myeloid and lymphoid cells.¹⁹⁸⁻²⁰⁰

The release of HMGB1 from cells succumbing to ICD requires the permeabilization of both the nuclear and plasma membranes, *de facto* constituting a *post-mortem* event.^{3,41} Although autophagy has been proposed to contribute to the release of HMGB1 from dying cells, at least under some circumstances,²⁰¹ the molecular machinery that underlies this crucial manifestation of ICD has not yet been elucidated in detail. This said, extracellular HMGB1 is well known to mediate robust pro-inflammatory effects upon binding to several receptors on the surface of immune cells, including TLR2, TLR4 and advanced glycosylation end product-specific receptor (AGER, best known as RAGE).²⁰²⁻²¹⁰ Moreover, extracellular HMGB1 reportedly exerts a chemotactic activity by forming a complex with chemokine (C-X-C motif) ligand 12 (CXCL12) that signals via chemokine (C-X-C motif) receptor 4 (CXCR4).²¹¹ Finally, at least under some circumstances, endogenous HMGB1 appears to promote autophagy by interfering with the mutually inhibitory interaction between the central autophagic regulator beclin 1 (BECN1) and the anti-apoptotic protein B-cell CLL/lymphoma 2 (BCL2).²¹²⁻²¹⁴ It is therefore tempting to speculate, yet remains to be formally demonstrated, that the nuclear release of HMGB1 may contribute to the autophagic response of cells succumbing to ICD inducers. Of note, the biological activity of extracellular HMGB1 appears to be regulated by its redox state.²¹⁵⁻²²¹ Moreover, HMGB1 binds not only to TLR2, TLR4 and RAGE, but also to hepatitis A virus cellular receptor 2 (HAVCR2, best known as TIM-3), hence mediating immunosuppressive (as opposed to immunostimulatory) effects.²²²⁻²²⁴ Taken together, these observations suggest that the biological activity of HMGB1 exhibits a consistent-degree of context-dependency. Nonetheless, HMGB1-deficient malignant cells exposed to ICD inducers fail to elicit adaptive immune responses upon inoculation into immunocompetent syngeneic mice, a defect that can be corrected by the co-administration of synthetic TLR4 ligands.²²⁵⁻²²⁸ Together with the notion that *Tlr4*^{-/-} mice fail to perceive anthracycline-treated syngeneic cells as

immunogenic,^{41,229} this observation demonstrates the importance of the HMGB1-TLR4 signaling axis for ICD.

In summary, the spatiotemporally coordinated emission of specific DAMPs promotes the recruitment of APCs to sites of ongoing ICD, their ability to take up dead cell-derived particulate material, as well as their capacity to prime an adaptive immune response.⁶ This generally proceeds in two phases, involving the sequential recruitment and activation of IL-17-secreting $\gamma\delta$ T cells and $\alpha\beta$ CTLs.^{31,230} The latter not only mediate direct antineoplastic effects, mostly by secreting interferon γ (IFN γ) and via the granzyme-perforin pathway, but also underlie the establishment of protective immunological memory (**Figure 1**).²³¹

Gold-standard methods to monitor ICD

As it stands, the gold-standard approach to evaluate the ability of a specific stimulus to cause *bona fide* ICD relies on vaccination assays.^{6,22,30} In this setting, malignant cells are exposed *in vitro* to the lethal stimulus of choice, thoroughly washed (to remove the stimulus), resuspended in an adequate volume of PBS, and then inoculated subcutaneously into the flank of immunocompetent syngeneic mice. One week later, living cells of the same type are introduced subcutaneously into the opposite flank, and mice are routinely monitored for the appearance of a palpable neoplastic lesion (**Figure 2A**). The proportion of mice that do not develop subcutaneous tumors reflects the degree of immunogenicity of cell death as induced by the lethal trigger under evaluation. As a note, murine cells succumbing to prototypic inducers of ICD such as doxorubicin and mitoxantrone effectively vaccinate 80% of mice.^{34,95,232}

As a confirmatory assay, putative ICD inducers can be assessed for their ability to mediate immune system-dependent therapeutic effects against established neoplastic lesions.^{6,34,233} In this scenario, grafted, genetically-driven or chemically-induced subcutaneous or orthotopic tumors are established in both immunocompetent and immunodeficient mice. Malignant lesions are then allowed to progress until a pre-determined size or time point, beyond which tumor-bearing mice are treated with the compound under evaluation (**Figure 2B**). In this experimental setup, *bona fide* ICD inducers mediate optimal therapeutic effects in immunocompetent, but not in immunodeficient, mice.^{34,41,95,119,233} Importantly, this latter approach is suitable to validate the results of vaccination experiments but cannot be employed alone to determine the capacity of a specific intervention to cause ICD. Indeed, even the activity of antineoplastic regimens that fail to render dying cells immunogenic but induce other immunostimulatory effects is negatively affected by the absence of a functional immune system.^{104,105} Among other molecules, this applies to the microtubular inhibitor paclitaxel and the nucleoside analogue gemcitabine.^{104,105}

The main drawbacks of these types of assay relate to the use of rodents and syngeneic tumor models: the need for a tightly controlled sterile facility (which is mandatory for working with immunodeficient animals), prolonged times for the establishment/growth of neoplastic lesions, and significant costs. Moreover, vaccination and therapeutic tests for the detection of ICD are limited by the relatively restricted number of syngeneic tumor models that are currently available. Thus, although they constitute the gold-standard approach for the detection of ICD, vaccination assays relying on immunocompetent mice and syngeneic cancer cells are intrinsically incompatible with large screening campaigns. To circumvent this issue, various techniques that allow for the detection of one or more ICD manifestations *in vitro* and *in vivo* have been developed.^{6,234} Monitoring the immunostimulatory activity of lead compounds (be it linked to the induction of ICD or reflecting other mechanisms) early in the drug discovery pipeline may indeed speed up significantly the development of novel anticancer agents.¹⁰⁴

Detection of surrogate ICD biomarkers

A relatively ample panel of ICD-associated phenomena can be monitored *in vitro* to obtain insights into the ability of a specific intervention to provoke ICD (**Table 1**).

Cell death. By definition, ICD inducers must be cytotoxic and provoke cell death above a minimal threshold level. Cancer cells emit indeed a wide panel of DAMPs in response to non-lethal perturbations of homeostasis. However, such DAMPs differ in both qualitative and quantitative terms from those emitted by cells of the same type dying in response to the same stimulus applied with a lethal intensity/duration. Living cells are less likely to be taken up by APCs and ignite an adaptive immune response than their dying counterparts. Moreover, if the fraction of dying cells is excessively low, neoplastic lesions develop at the vaccination site and protective immunity cannot be established.^{34,95} Thus, agents that stimulate all the key manifestations of ICD including CALR exposure, ATP secretion and HMGB1 release, but fail to exert robust cytotoxic effects cannot be considered as authentic ICD inducers. This is the case of cardiac glycosides including digoxin and digitoxin, which nonetheless are powerful at converting non-immunogenic instances of cell death into *bona fide* ICD, hence operating as potent immune adjuvant.^{95-97,235}

Several assays are commercially available to monitor cell death-associated parameters, the most reliable indicator of cell death being end-stage plasma membrane permeabilization.^{9,236} This can be conveniently monitored by so-called exclusion dyes like the DNA-binding chemicals propidium iodide (PI) and 4',6-diamidino-2-phenylindole (DAPI), which only accumulate in cells with permeabilized plasma membranes. PI and DAPI can be conveniently detected by flow cytometry or fluorescence microscopy (absorption/emission peaks: 535/617 and 358/461 nm, respectively). On flow cytometry, both PI and DAPI can be combined with fluorescence variants of the protein annexin A5 (ANXA5), permitting the detection of phosphatidylserine exposure,^{9,237,238} as well as with 3,3'-dihexyloxacarbocyanine iodide (DiOC₆(3), absorption/emission peaks: 482/504 nm),

allowing for the quantification of mitochondrial transmembrane potential ($\Delta\psi_m$).²³⁹⁻²⁴¹ The externalization of phosphatidylserine (a phospholipid normally restricted to the inner leaflet of the plasma membrane) accompanies indeed multiple (though not all) instances of apoptotic cell death,^{16,242-245} while the permanent dissipation of the $\Delta\psi_m$ as a result of mitochondrial outer membrane permeabilization (MOMP) constitutes one of the major hallmarks of mitochondrial apoptosis.^{17,18,246,247} Of note, DiOC₆(3) is not compatible with fixation, but other $\Delta\psi_m$ -sensitive probes that exist are, including chloromethyltetramethylrosamine (absorption/emission peaks: 554/576 nm).²⁴⁸ MOMP is accompanied by the massive activation of caspase-9 and -3, while caspase-8 is required for ICD upstream of MOMP. The activation of caspases can be documented by flow cytometry or fluorescence microscopy, either upon the immunostaining of cells with monoclonal antibodies specific for active caspase fragments, or with cell-permeant caspase substrates that become fluorescent upon cleavage.^{9,249,250} Alternatively, caspase activation can be detected in a semi-quantitative manner by immunoblotting, with antibodies specific for caspases (which are themselves activated by cleavage) or their substrates.^{250,251}

As MOMP ensues the assembly of BAX/BAK1-containing oligomers across the outer mitochondrial membrane, the process can also be monitored by means of green fluorescent protein (GFP)-BAX chimeras (GFP absorption/emission peaks: 395/509 nm). In this setting, the relocalization of BAX to mitochondria can be followed by fluorescence microscopy as a shift in GFP fluorescence from a diffuse to a punctate or network-like pattern.^{40,252} Finally, one of the major morphological modifications of apoptosis (and hence of ICD) is nuclear condensation (pyknosis).^{1,2,95} Also this process can be conveniently monitored by fluorescence microscopy, either in cells that constitutively express a GFP- or red fluorescent protein (RFP)-tagged variant of histone 2B (RFP-H2B, absorption/emission peaks: 584/607 nm) or upon fixation and staining with the chromatinophilic dye Hoechst 33342 (absorption/emission peaks: 361/461 nm).^{40,95,235}

CALR exposure. Several assays are available to directly monitor the ICD-associated translocation of CALR on the outer leaflet of the plasma membrane. For instance, this can be achieved on flow cytometry, by staining non-permeabilized cells with a CALR-specific antibody, or in cells that stably express a CALR-HaloTagTM fusion protein.^{40,95} In the latter scenario, the HaloTagTM label can be visualized by a cell-impermeant fluorescent chemical, resulting in the specific detection of the CALR molecules that are effectively accessible for ligand binding from the extracellular microenvironment.^{40,95} In both cases, it is imperative to remove from the analysis dead (PI⁺ or DAPI⁺) cells, as the permeabilized plasma membrane allows both the CALR-specific antibody and the normally cell-impermeant HaloTagTM ligand to access intracellular CALR.^{34,40} Alternatively, CALR exposure can be monitored upon the biotinylation of cell surface proteins (which must be performed in pre-apoptotic conditions, when plasma membranes are intact, to avoid false-positive results owing to intracellular CALR), followed by streptavidin-mediated precipitation, and detection by immunoblotting,^{34,66,253} or by fluorescence microscopy, in cells that constitutively express a CALR-GFP fusion construct. For the sake of precision, it should be noted that the latter system does not detect CALR-GFP exposure in itself, but the ER perinuclear clustering that invariably accompanies exposure.^{20,232} We have also successfully employed a PDIA3-specific antibody and flow cytometry as well as PDIA3-GFP-expressing cells and fluorescence microscopy to (indirectly) assess CALR exposure in the course of ICD, as in our models PDIA3 invariably co-translocates with CALR on the surface of cells exposed to ICD inducers.^{36,37,95} However, this does not apply to all experimental settings,^{66,67} implying that the PDIA3-GFP fusion is a useful confirmatory tool but cannot be employed as a standalone means to identify all instances of ICD.

In some instances, it may be important to monitor CALR exposure along with the proficiency of the ER stress response. This may indeed allow for the identification of defects in the signaling pathway that leads to the translocation of CALR to the outer leaflet of the plasma membrane. Several assays are currently available for the detection of the different arms of the ER stress response.^{136,254-256} For

instance, the phosphorylation state of EIF2A and/or of the major EIF2A kinases, including EIF2A kinase 1 (EIF2AK1, best known as HRI),²⁵⁷ EIF2AK2 (best known as PKR),²⁵⁸ and EIF2AK3 (best known as PERK),²⁵⁹⁻²⁶¹ can be assessed by immunoblotting, flow cytometry or immunofluorescence microscopy with phosphoepitope-specific antibodies.²⁶⁰ The splicing status of X-box binding protein 1 (*XBPI*) mRNA, reflecting the activation of the ER stress sensor endoplasmic reticulum to nucleus signaling 1 (ERN1, best known as IRE1 α), can be monitored by quantitative real-time RT-PCR,²⁶² as well as by flow cytometry or fluorescence microscopy, either in cells that express a fluorescently-tagged version of XBPI²⁶³ or upon the administration of a self-quenched RNA probe that can be cleaved by IRE1 α .²⁶⁴ Finally, the nuclear redistribution of activating transcription factor 6 (ATF6) can be easily evaluated by fluorescence microscopy in cells that constitutively express GFP- or RFP-tagged variants of ATF6.⁵² As an alternative, ER stress can be indirectly monitored upon the formation of GTPase activating protein (SH3 domain) binding protein 1 (G3BP1)-containing granules in cells genetically modified to express a G3BP1-GFP fusion.^{40,265} This said, G3BP1 appears to redistribute to granules in response to a wide panel of stressful conditions that are not limited to specific perturbations of reticular homeostasis. Thus, monitoring G3BP1 aggregation can be useful to determine whether cells mount a stress response to a putative inducer of ICD, yet cannot be employed to formally imply the ER in this process.

ATP secretion. The ICD-associated secretion of ATP can be monitored by two complementary approaches: directly, by quantification of extracellular ATP,^{137,180} or indirectly, by the assessment of residual intracellular ATP.^{137,139} The most employed method currently available for the quantification of ATP levels relies on the ability of eukaryotic luciferases to produce light while oxidizing *D*-(-)-luciferin (which must be added exogenously) in a ATP-dependent manner.^{266,267} This can be applied to culture supernatants as well as to cell lysates, and hence is compatible with both the direct and indirect assessment of ATP secretion in the course of ICD. The vesicular pool of

ATP can also be visualized by fluorescence microscopy upon staining cells with the ATP-binding fluorochrome quinacrine (absorption/emission peaks: 436/525 nm).²⁶⁸ Alternatively, intracellular ATP can be monitored in living cells by a fluorescence resonance energy transfer (FRET)-based assay involving a yellow fluorescent protein-cyan fluorescent protein (YFP-CFP) fusion containing a domain that changes its conformation upon ATP binding, hence shifting the spectral properties of the probe.²⁶⁹

In some settings, it may be relevant to monitor the autophagic response that generally precedes and is required for ICD-associated ATP release. This can be achieved by a wide panel of techniques, whose detailed discussion goes largely beyond the scope of this set of recommendations.^{15,270,271} This said, one of the most convenient approaches to obtain insights into the autophagic response of cells exposed to homeostatic perturbations relies on the use of a GFP- or RFP-tagged variant of microtubule-associated protein 1 light chain 3 (MAP1LC3, best known as LC3).²⁷² In the course of autophagy, LC3 gets conjugated to phosphatidylethanolamine, hence acquiring the ability to accumulate into forming autophagosomes.^{273,274} In line with this notion, GFP-LC3 redistributes from a diffuse to a punctate pattern in cells mounting an autophagic response, a phenomenon that can readily be monitored by fluorescence microscopy.

HMGB1 release. Similar to the secretion of ATP, the release of HMGB1 in the supernatant of cells undergoing ICD can be monitored directly or indirectly, as a function of residual intracellular HMGB1.^{41,207,275} The former approach relies on the immunoblotting-based assessment of HMGB1 in concentrated cell supernatants, or (most often) on commercially available enzyme-linked immunosorbent assay (ELISA) kits specific for human or murine HMGB1. These kits generally allow for the precise quantification of HMGB1 concentrations in a wide panel of biological specimens, including culture supernatants, serum samples and interstitial fluids, yet may be relatively expensive for use in large-scale screening campaigns.^{95,147,275} Alternatively, HMGB1

release can be assessed by fluorescence microscopy in cells expressing a GFP-tagged variant of HMGB1, as the loss of colocalization between the GFP signal and a nuclear staining (e.g., Hoechst 33342, H2B-RFP).²⁷⁵ This said, the precise quantification of HMGB1 variants exhibiting differential redox states requires mass spectroscopy.²⁷⁶

High-content, high-throughput platform. Cell death that is not accompanied by CALR exposure, ATP secretion and HMGB1 release is generally not perceived as immunogenic.^{34,41,119} In other words, the absence of only one such ICD-associated events often entails a consistent decrease in the immunogenicity of cell death, if not its total loss. This implies that the ability of a given intervention to promote ICD can be inferred *in vitro* only upon the concurrent evaluation of all ICD hallmarks. Indeed, cells succumbing to homeostatic perturbations that stimulate ATP secretion and HMGB1 release but not CALR exposure, such as the administration of cisplatin, fail to elicit adaptive immune responses upon inoculation into immunocompetent mice.^{34,39,40} This said, a platform that would allow for the simultaneous detection of cell death, CALR exposure, ATP secretion and HMGB1 release in the context of large screening campaigns was missing. To circumvent this obstacle to the identification of novel, perhaps clinically relevant *bona fide* inducers of ICD, we recently developed a robotized cell biology platform that allows for entirely automated compound handling and multiplex read-out capability (including fluorescence microscopy, flow cytometry and bioluminescence detection) in sterile conditions. We then designed fully automated workflows based on various combinations of the assays described above and including appropriate procedures for data handling/normalization and statistical analysis. This approach is compatible with the high-content, high-throughput screening of large chemical libraries, returning a cumulative score that represents the ability of a specific compound to promote the four tenets of ICD. Importantly, this integrated platform does not abolish the need to evaluate putative ICD inducers for their capacity to elicit protective anticancer immune responses in gold-standard vaccination assays.

Nonetheless, it allows for the relatively straightforward identification of candidate molecules. By means of this approach, septacidin has been identified as a *bona fide* ICD inducer.²³² Moreover, cardiac glycosides were found to robustly improve the immunogenic potential of cell death.^{95-97,235} We expect this platform not only to allow for the discovery of other ICD inducers, but also to facilitate the understanding of the molecular pathways that underlie ICD and how these can be modulated for therapeutic purposes.

Concluding remarks and future directions

As described above, the simultaneous detection of cell death, CALR exposure, ATP secretion and HMGB1 release by means of a high-content-, high-throughput-compatible platform is useful for the identification of candidate ICD inducers among large chemical libraries. Nonetheless, vaccination assays involving immunocompetent mice and syngeneic cancer cells do not cease to constitute the gold-standard approach to formally identify *bona fide* triggers of ICD.

Paradoxically, the major obstacle to the identification and development of clinically relevant ICD inducers appears to be represented by the murine system itself, as rodent and human cells do not necessarily respond to a specific stimulus in a comparable fashion. As a standalone example, mouse cells are highly resistant to the cytotoxic activity of cardiac glycosides, owing to the expression of a mutated subunit of their target, the Na²⁺/K⁺ ATPase.^{95,277} This implies that formally determining whether a given intervention provokes ICD in the human system is complicated. Humanized rodent models, i.e., immunodeficient mice reconstituted with a human immune system,²⁷⁸ may partially circumvent this issue. However, the interaction between human immune cells and the murine microenvironment may be negatively influenced by inter-species molecular variations that compromise the ability of the former to mount an appropriate immune response.^{279,280} Thus, although attempts are being made to limit such variations,²⁸¹ experimental models that allow for the proper evaluation of ICD in the human system require further improvements. Finally, the procedure outlined above for the identification of novel ICD inducers assesses the biochemical processes that are required for the immunogenicity of anthracycline-induced cell death. However, ICD might exist in functionally distinct variants, implying that hitherto uncharacterized mechanisms might render cell death immunogenic. This possibility should be actively investigated in future studies.

Irrespective of these caveats, we are confident that the screening of large chemical or small-interfering RNA libraries combined with vaccination assays in the murine model will allow for the

identification of novel, therapeutically relevant interventions for the induction or modulation of ICD. Moreover, the immunohistochemical detection of ICD-associated biomarkers in bioptic specimens from cancer patients may convey robust predictive or prognostic indications, at least under some circumstances.^{282,283} The implementation of well-designed, longitudinal immunomonitoring procedures into the clinical development of antineoplastic agents is required to ascertain the actual prognostic or predictive value of ICD-associated processes among oncological patients.²⁸⁴⁻²⁸⁶ Of note, a phase I clinical study has recently been launched to investigate the safety and preliminary therapeutic efficacy of adenoviral vectors genetically modified to trigger ICD, in subjects with malignant glioma and glioblastoma multiforme (NCT01811992). In this setting, serotype 5, replication-defective, first-generation adenoviruses encoding the HSV-1 thymidine kinase and similar vectors coding for FLT3LG are co-infused at the time of surgical tumor resection, followed by valacyclovir (a gancyclovir-like prodrug converted by the viral thymidine kinase and cellular kinases into its triphosphate cytotoxic variant)^{287,288} in the context of current standard-of-care therapy (source <https://clinicaltrials.gov/>). The results of such a first-in-man study relying on the genetic induction of ICD in cancer patients are urgently awaited.

Acknowledgements. *GL and LG are supported by the Ligue contre le Cancer (équipe labellisée); Agence National de la Recherche (ANR); Association pour la recherche sur le cancer (ARC); Cancéropôle Ile-de-France; AXA Chair for Longevity Research; Institut National du Cancer (INCa); Fondation Bettencourt-Schueller; Fondation de France; Fondation pour la Recherche Médicale (FRM); the European Commission (ArtForce); the European Research Council (ERC); the LabEx Immuno-Oncology; the SIRIC Stratified Oncology Cell DNA Repair and Tumor Immune Elimination (SOCRATE); the SIRIC Cancer Research and Personalized Medicine (CARPEM); and the Paris Alliance of Cancer Research Institutes (PACRI).*

References

1. Galluzzi L, Maiuri MC, Vitale I, Zischka H, Castedo M, Zitvogel L, et al. Cell death modalities: classification and pathophysiological implications. *Cell Death Differ* 2007; 14:1237-43.
2. Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Baehrecke EH, et al. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. *Cell Death Differ* 2009; 16:3-11.
3. Galluzzi L, Vitale I, Abrams JM, Alnemri ES, Baehrecke EH, Blagosklonny MV, et al. Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. *Cell Death Differ* 2012; 19:107-20.
4. Vandenabeele P, Galluzzi L, Vanden Berghe T, Kroemer G. Molecular mechanisms of necroptosis: an ordered cellular explosion. *Nat Rev Mol Cell Biol* 2010; 11:700-14.
5. Vanden Berghe T, Linkermann A, Jouan-Lanhouet S, Walczak H, Vandenabeele P. Regulated necrosis: the expanding network of non-apoptotic cell death pathways. *Nat Rev Mol Cell Biol* 2014; 15:135-47.
6. Kroemer G, Galluzzi L, Kepp O, Zitvogel L. Immunogenic cell death in cancer therapy. *Annu Rev Immunol* 2013; 31:51-72.
7. Krysko DV, Garg AD, Kaczmarek A, Krysko O, Agostinis P, Vandenabeele P. Immunogenic cell death and DAMPs in cancer therapy. *Nat Rev Cancer* 2012; 12:860-75.
8. Vogt CI. Untersuchungen über die Entwicklungsgeschichte der Geburtshelferkröte (*Alytes obstetricans*). Jent, 1842.
9. Galluzzi L, Aaronson SA, Abrams J, Alnemri ES, Andrews DW, Baehrecke EH, et al. Guidelines for the use and interpretation of assays for monitoring cell death in higher eukaryotes. *Cell Death Differ* 2009; 16:1093-107.
10. Galluzzi L, Vitale I, Michels J, Brenner C, Szabadkai G, Harel-Bellan A, et al. Systems biology of cisplatin resistance: past, present and future. *Cell Death Dis* 2014; 5:e1257.

11. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 1972; 26:239-57.
12. Levine B, Yuan J. Autophagy in cell death: an innocent convict? *J Clin Invest* 2005; 115:2679-88.
13. Ericsson JL. Studies on induced cellular autophagy. I. Electron microscopy of cells with in vivo labelled lysosomes. *Exp Cell Res* 1969; 55:95-106.
14. Maximow AA. Studies on the Changes Produced by Roentgen Rays in Inflamed Connective Tissue. *J Exp Med* 1923; 37:319-40.
15. Klionsky DJ, Abeliovich H, Agostinis P, Agrawal DK, Aliev G, Askew DS, et al. Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. *Autophagy* 2008; 4:151-75.
16. Martin SJ, Reutelingsperger CP, McGahon AJ, Rader JA, van Schie RC, LaFace DM, et al. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J Exp Med* 1995; 182:1545-56.
17. Tait SW, Green DR. Mitochondria and cell death: outer membrane permeabilization and beyond. *Nat Rev Mol Cell Biol* 2010; 11:621-32.
18. Kroemer G, Galluzzi L, Brenner C. Mitochondrial membrane permeabilization in cell death. *Physiol Rev* 2007; 87:99-163.
19. Taylor RC, Cullen SP, Martin SJ. Apoptosis: controlled demolition at the cellular level. *Nat Rev Mol Cell Biol* 2008; 9:231-41.
20. Candi E, Schmidt R, Melino G. The cornified envelope: a model of cell death in the skin. *Nat Rev Mol Cell Biol* 2005; 6:328-40.
21. Galluzzi L, Kepp O, Krautwald S, Kroemer G, Linkermann A. Molecular mechanisms of regulated necrosis. *Semin Cell Dev Biol* 2014; IN PRESS.
22. Casares N, Pequignot MO, Tesniere A, Ghiringhelli F, Roux S, Chaput N, et al. Caspase-dependent immunogenicity of doxorubicin-induced tumor cell death. *J Exp Med* 2005; 202:1691-701.

23. Cirone M, Di Renzo L, Lotti LV, Conte V, Trivedi P, Santarelli R, et al. Activation of dendritic cells by tumor cell death. *Oncoimmunology* 2012; 1:1218-9.
24. Henson PM, Hume DA. Apoptotic cell removal in development and tissue homeostasis. *Trends Immunol* 2006; 27:244-50.
25. Abud HE. Shaping developing tissues by apoptosis. *Cell Death Differ* 2004; 11:797-9.
26. Baumann I, Kolowos W, Voll RE, Manger B, Gaip U, Neuhuber WL, et al. Impaired uptake of apoptotic cells into tingible body macrophages in germinal centers of patients with systemic lupus erythematosus. *Arthritis Rheum* 2002; 46:191-201.
27. Munoz LE, Lauber K, Schiller M, Manfredi AA, Herrmann M. The role of defective clearance of apoptotic cells in systemic autoimmunity. *Nat Rev Rheumatol* 2010; 6:280-9.
28. Vacchelli E, Galluzzi L, Fridman WH, Galon J, Sautes-Fridman C, Tartour E, et al. Trial watch: Chemotherapy with immunogenic cell death inducers. *Oncoimmunology* 2012; 1:179-88.
29. Vacchelli E, Senovilla L, Eggermont A, Fridman WH, Galon J, Zitvogel L, et al. Trial watch: Chemotherapy with immunogenic cell death inducers. *Oncoimmunology* 2013; 2:e23510.
30. Vacchelli E, Aranda F, Eggermont A, Galon J, Sautes-Fridman C, Cremer I, et al. Trial Watch: Chemotherapy with immunogenic cell death inducers. *Oncoimmunology* 2014; 3:e27878.
31. Mattarollo SR, Loi S, Duret H, Ma Y, Zitvogel L, Smyth MJ. Pivotal role of innate and adaptive immunity in anthracycline chemotherapy of established tumors. *Cancer Res* 2011; 71:4809-20.
32. Cirone M, Garufi A, Di Renzo L, Granato M, Faggioni A, D'Orazi G. Zinc supplementation is required for the cytotoxic and immunogenic effects of chemotherapy in chemoresistant p53-functionally deficient cells. *Oncoimmunology* 2013; 2:e26198.
33. Bracci L, Schiavoni G, Sistigu A, Belardelli F. Immune-based mechanisms of cytotoxic chemotherapy: implications for the design of novel and rationale-based combined treatments against cancer. *Cell Death Differ* 2014; 21:15-25.

34. Obeid M, Tesniere A, Ghiringhelli F, Fimia GM, Apetoh L, Perfettini JL, et al. Calreticulin exposure dictates the immunogenicity of cancer cell death. *Nat Med* 2007; 13:54-61.
35. Fucikova J, Kralikova P, Fialova A, Brtnicky T, Rob L, Bartunkova J, et al. Human tumor cells killed by anthracyclines induce a tumor-specific immune response. *Cancer Res* 2011; 71:4821-33.
36. Panaretakis T, Kepp O, Brockmeier U, Tesniere A, Bjorklund AC, Chapman DC, et al. Mechanisms of pre-apoptotic calreticulin exposure in immunogenic cell death. *EMBO J* 2009; 28:578-90.
37. Panaretakis T, Joza N, Modjtahedi N, Tesniere A, Vitale I, Durchschlag M, et al. The co-translocation of ERp57 and calreticulin determines the immunogenicity of cell death. *Cell Death Differ* 2008; 15:1499-509.
38. Bugaut H, Bruchard M, Berger H, Derangere V, Odoul L, Euvrard R, et al. Bleomycin exerts ambivalent antitumor immune effect by triggering both immunogenic cell death and proliferation of regulatory T cells. *PLoS One* 2013; 8:e65181.
39. Tesniere A, Schlemmer F, Boige V, Kepp O, Martins I, Ghiringhelli F, et al. Immunogenic death of colon cancer cells treated with oxaliplatin. *Oncogene* 2010; 29:482-91.
40. Martins I, Kepp O, Schlemmer F, Adjemian S, Tailler M, Shen S, et al. Restoration of the immunogenicity of cisplatin-induced cancer cell death by endoplasmic reticulum stress. *Oncogene* 2011; 30:1147-58.
41. Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A, et al. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. *Nat Med* 2007; 13:1050-9.
42. Gou HF, Huang J, Shi HS, Chen XC, Wang YS. Chemo-immunotherapy with oxaliplatin and interleukin-7 inhibits colon cancer metastasis in mice. *PLoS One* 2014; 9:e85789.
43. Tongu M, Harashima N, Yamada T, Harada T, Harada M. Immunogenic chemotherapy with cyclophosphamide and doxorubicin against established murine carcinoma. *Cancer Immunol Immunother* 2010; 59:769-77.

44. Schiavoni G, Sistigu A, Valentini M, Mattei F, Sestili P, Spadaro F, et al. Cyclophosphamide synergizes with type I interferons through systemic dendritic cell reactivation and induction of immunogenic tumor apoptosis. *Cancer Res* 2011; 71:768-78.
45. Sistigu A, Viaud S, Chaput N, Bracci L, Proietti E, Zitvogel L. Immunomodulatory effects of cyclophosphamide and implementations for vaccine design. *Semin Immunopathol* 2011; 33:369-83.
46. Stoetzer OJ, Fersching DM, Salat C, Steinkohl O, Gabka CJ, Hamann U, et al. Circulating immunogenic cell death biomarkers HMGB1 and RAGE in breast cancer patients during neoadjuvant chemotherapy. *Tumour Biol* 2013; 34:81-90.
47. Chen X, Yang Y, Zhou Q, Weiss JM, Howard OZ, McPherson JM, et al. Effective chemoimmunotherapy with anti-TGFbeta antibody and cyclophosphamide in a mouse model of breast cancer. *PLoS One* 2014; 9:e85398.
48. Guerriero JL, Ditsworth D, Fan Y, Zhao F, Crawford HC, Zong WX. Chemotherapy induces tumor clearance independent of apoptosis. *Cancer Res* 2008; 68:9595-600.
49. Beneteau M, Zunino B, Jacquin MA, Meynet O, Chiche J, Pradelli LA, et al. Combination of glycolysis inhibition with chemotherapy results in an antitumor immune response. *Proc Natl Acad Sci U S A* 2012; 109:20071-6.
50. Galluzzi L, Kepp O, Vander Heiden MG, Kroemer G. Metabolic targets for cancer therapy. *Nat Rev Drug Discov* 2013; 12:829-46.
51. Hoffmann J, Vitale I, Buchmann B, Galluzzi L, Schwede W, Senovilla L, et al. Improved cellular pharmacokinetics and pharmacodynamics underlie the wide anticancer activity of sagopilone. *Cancer Res* 2008; 68:5301-8.
52. Senovilla L, Vitale I, Martins I, Tailler M, Pailleret C, Michaud M, et al. An immunosurveillance mechanism controls cancer cell ploidy. *Science* 2012; 337:1678-84.
53. Pellicciotta I, Yang CP, Goldberg GL, Shahabi S. Epothilone B enhances Class I HLA and HLA-A2 surface molecule expression in ovarian cancer cells. *Gynecol Oncol* 2011; 122:625-31.

54. Dutcher JD, Vonsaltza MH, Pansy FE. Septacidin, a New Antitumor and Antifungal Antibiotic Produced by *Streptomyces Fibriatus*. *Antimicrob Agents Chemother* (Bethesda) 1963; 161:83-8.
55. Sukkurwala AQ, Martins I, Wang Y, Schlemmer F, Ruckenstein C, Durchschlag M, et al. Immunogenic calreticulin exposure occurs through a phylogenetically conserved stress pathway involving the chemokine CXCL8. *Cell Death Differ* 2014; 21:59-68.
56. Perez CA, Fu A, Onishko H, Hallahan DE, Geng L. Radiation induces an antitumour immune response to mouse melanoma. *Int J Radiat Biol* 2009; 85:1126-36.
57. Vacchelli E, Vitale I, Tartour E, Eggermont A, Sautes-Fridman C, Galon J, et al. Trial Watch: Anticancer radioimmunotherapy. *Oncoimmunology* 2013; 2:e25595.
58. Galluzzi L, Kepp O, Kroemer G. Immunogenic cell death in radiation therapy. *Oncoimmunology* 2013; 2:e26536.
59. Suzuki Y, Mimura K, Yoshimoto Y, Watanabe M, Ohkubo Y, Izawa S, et al. Immunogenic tumor cell death induced by chemoradiotherapy in patients with esophageal squamous cell carcinoma. *Cancer Res* 2012; 72:3967-76.
60. Ko A, Kanehisa A, Martins I, Senovilla L, Chargari C, Dugue D, et al. Autophagy inhibition radiosensitizes in vitro, yet reduces radioresponses in vivo due to deficient immunogenic signalling. *Cell Death Differ* 2014; 21:92-9.
61. Formenti SC, Demaria S. Radiation therapy to convert the tumor into an in situ vaccine. *Int J Radiat Oncol Biol Phys* 2012; 84:879-80.
62. Gameiro SR, Jammeh ML, Wattenberg MM, Tsang KY, Ferrone S, Hodge JW. Radiation-induced immunogenic modulation of tumor enhances antigen processing and calreticulin exposure, resulting in enhanced T-cell killing. *Oncotarget* 2014; 5:403-16.
63. Schildkopf P, Frey B, Ott OJ, Rubner Y, Multhoff G, Sauer R, et al. Radiation combined with hyperthermia induces HSP70-dependent maturation of dendritic cells and release of pro-inflammatory cytokines by dendritic cells and macrophages. *Radiother Oncol* 2011; 101:109-15.
64. Gorin JB, Menager J, Gouard S, Maurel C, Guilloux Y, Faivre-Chauvet A, et al. Antitumor immunity induced after alpha irradiation. *Neoplasia* 2014; 16:319-28.

65. Garg AD, Krysko DV, Vandenabeele P, Agostinis P. Hypericin-based photodynamic therapy induces surface exposure of damage-associated molecular patterns like HSP70 and calreticulin. *Cancer Immunol Immunother* 2012; 61:215-21.
66. Garg AD, Krysko DV, Verfaillie T, Kaczmarek A, Ferreira GB, Marysael T, et al. A novel pathway combining calreticulin exposure and ATP secretion in immunogenic cancer cell death. *EMBO J* 2012; 31:1062-79.
67. Galluzzi L, Kepp O, Kroemer G. Enlightening the impact of immunogenic cell death in photodynamic cancer therapy. *EMBO J* 2012; 31:1055-7.
68. Garg AD, Dudek AM, Ferreira GB, Verfaillie T, Vandenabeele P, Krysko DV, et al. ROS-induced autophagy in cancer cells assists in evasion from determinants of immunogenic cell death. *Autophagy* 2013; 9:1292-307.
69. Garg AD, Agostinis P. ER stress, autophagy and immunogenic cell death in photodynamic therapy-induced anti-cancer immune responses. *Photochem Photobiol Sci* 2014; 13:474-87.
70. Garg AD, Dudek AM, Agostinis P. Autophagy-dependent suppression of cancer immunogenicity and effector mechanisms of innate and adaptive immunity. *Oncoimmunology* 2013; 2:e26260.
71. Korbelik M, Sun J, Cecic I. Photodynamic therapy-induced cell surface expression and release of heat shock proteins: relevance for tumor response. *Cancer Res* 2005; 65:1018-26.
72. Panzarini E, Inguscio V, Dini L. Immunogenic cell death: can it be exploited in PhotoDynamic Therapy for cancer? *Biomed Res Int* 2013; 2013:482160.
73. Yu P, Fu YX. Targeting tumors with LIGHT to generate metastasis-clearing immunity. *Cytokine Growth Factor Rev* 2008; 19:285-94.
74. Fucikova J, Moserova I, Truxova I, Hermanova I, Vancurova I, Partlova S, et al. High hydrostatic pressure induces immunogenic cell death in human tumor cells. *Int J Cancer* 2014; 135:1165-77.
75. Pol J, Bloy N, Obrist F, Eggermont A, Galon J, Cremer I, et al. Trial Watch: Oncolytic viruses for cancer therapy. *Oncoimmunology* 2014; 3:28694.

76. Vacchelli E, Eggermont A, Sautes-Fridman C, Galon J, Zitvogel L, Kroemer G, et al. Trial watch: Oncolytic viruses for cancer therapy. *Oncoimmunology* 2013; 2:e24612.
77. Angelova AL, Grekova SP, Heller A, Kuhlmann O, Soyka E, Giese T, et al. Complementary induction of immunogenic cell death by oncolytic parvovirus H-1PV and gemcitabine in pancreatic cancer. *J Virol* 2014; 88:5263-76.
78. Workenhe ST, Mossman KL. Rewiring cancer cell death to enhance oncolytic viro-immunotherapy. *Oncoimmunology* 2013; 2:e27138.
79. Workenhe ST, Pol JG, Lichty BD, Cummings DT, Mossman KL. Combining oncolytic HSV-1 with immunogenic cell death-inducing drug mitoxantrone breaks cancer immune tolerance and improves therapeutic efficacy. *Cancer Immunol Res* 2013; 1:309-19.
80. Bartlett DL, Liu Z, Sathiaiah M, Ravindranathan R, Guo Z, He Y, et al. Oncolytic viruses as therapeutic cancer vaccines. *Mol Cancer* 2013; 12:103.
81. Guo ZS, Liu Z, Bartlett DL. Oncolytic Immunotherapy: Dying the Right Way is a Key to Eliciting Potent Antitumor Immunity. *Front Oncol* 2014; 4:74.
82. Huang B, Sikorski R, Kirn DH, Thorne SH. Synergistic anti-tumor effects between oncolytic vaccinia virus and paclitaxel are mediated by the IFN response and HMGB1. *Gene Ther* 2011; 18:164-72.
83. Liikanen I, Ahtiainen L, Hirvinen ML, Bramante S, Cerullo V, Nokisalmi P, et al. Oncolytic adenovirus with temozolomide induces autophagy and antitumor immune responses in cancer patients. *Mol Ther* 2013; 21:1212-23.
84. Mineharu Y, King GD, Muhammad AK, Bannykh S, Kroeger KM, Liu C, et al. Engineering the brain tumor microenvironment enhances the efficacy of dendritic cell vaccination: implications for clinical trial design. *Clin Cancer Res* 2011; 17:4705-18.
85. Spisek R, Charalambous A, Mazumder A, Vesole DH, Jagannath S, Dhodapkar MV. Bortezomib enhances dendritic cell (DC)-mediated induction of immunity to human myeloma via exposure of cell surface heat shock protein 90 on dying tumor cells: therapeutic implications. *Blood* 2007; 109:4839-45.
86. Demaria S, Santori FR, Ng B, Liebes L, Formenti SC, Vukmanovic S. Select forms of tumor cell apoptosis induce dendritic cell maturation. *J Leukoc Biol* 2005; 77:361-8.

87. Cirone M, Di Renzo L, Lotti LV, Conte V, Trivedi P, Santarelli R, et al. Primary effusion lymphoma cell death induced by bortezomib and AG 490 activates dendritic cells through CD91. *PLoS One* 2012; 7:e31732.
88. Chen HM, Wang PH, Chen SS, Wen CC, Chen YH, Yang WC, et al. Shikonin induces immunogenic cell death in tumor cells and enhances dendritic cell-based cancer vaccine. *Cancer Immunol Immunother* 2012; 61:1989-2002.
89. Garrido G, Rabasa A, Sanchez B, Lopez MV, Blanco R, Lopez A, et al. Induction of immunogenic apoptosis by blockade of epidermal growth factor receptor activation with a specific antibody. *J Immunol* 2011; 187:4954-66.
90. D'Eliseo D, Manzi L, Velotti F. Capsaicin as an inducer of damage-associated molecular patterns (DAMPs) of immunogenic cell death (ICD) in human bladder cancer cells. *Cell Stress Chaperones* 2013; 18:801-8.
91. Gilardini Montani MS, D'Eliseo D, Cirone M, Di Renzo L, Faggioni A, Santoni A, et al. Capsaicin-mediated apoptosis of human bladder cancer cells activates dendritic cells via CD91. *Nutrition* 2014:IN PRESS.
92. Molinari R, D'Eliseo D, Manzi L, Zolla L, Velotti F, Merendino N. The n3-polyunsaturated fatty acid docosahexaenoic acid induces immunogenic cell death in human cancer cell lines via pre-apoptotic calreticulin exposure. *Cancer Immunol Immunother* 2011; 60:1503-7.
93. Emeagi PU, Van Lint S, Goyvaerts C, Maenhout S, Cauwels A, McNeish IA, et al. Proinflammatory characteristics of SMAC/DIABLO-induced cell death in antitumor therapy. *Cancer Res* 2012; 72:1342-52.
94. Emeagi PU, Thielemans K, Breckpot K. The role of SMAC mimetics in regulation of tumor cell death and immunity. *Oncoimmunology* 2012; 1:965-7.
95. Menger L, Vacchelli E, Adjemian S, Martins I, Ma Y, Shen S, et al. Cardiac glycosides exert anticancer effects by inducing immunogenic cell death. *Sci Transl Med* 2012; 4:143ra99.
96. Kepp O, Menger L, Vacchelli E, Adjemian S, Martins I, Ma Y, et al. Anticancer activity of cardiac glycosides: At the frontier between cell-autonomous and immunological effects. *Oncoimmunology* 2012; 1:1640-2.

97. Menger L, Vacchelli E, Kepp O, Eggermont A, Tartour E, Zitvogel L, et al. Trial watch: Cardiac glycosides and cancer therapy. *Oncoimmunology* 2013; 2:e23082.
98. Riganti C, Castella B, Kopecka J, Campia I, Coscia M, Pescarmona G, et al. Zoledronic acid restores doxorubicin chemosensitivity and immunogenic cell death in multidrug-resistant human cancer cells. *PLoS One* 2013; 8:e60975.
99. Riganti C, Massaia M. Inhibition of the mevalonate pathway to override chemoresistance and promote the immunogenic demise of cancer cells: Killing two birds with one stone. *Oncoimmunology* 2013; 2:e25770.
100. Liljenfeldt L, Gkirtzimanaki K, Vyrila D, Svensson E, Loskog AS, Eliopoulos AG. Enhanced therapeutic anti-tumor immunity induced by co-administration of 5-fluorouracil and adenovirus expressing CD40 ligand. *Cancer Immunol Immunother* 2014; 63:273-82.
101. Palombo F, Focaccetti C, Barnaba V. Therapeutic implications of immunogenic cell death in human cancer. *Front Immunol* 2014; 4:503.
102. Olsson L, Ebbesen P. Experimental induction of tumor growth control by immune adjuvants: current status and some theories to be explored. *Biomedicine* 1978; 28:88-91.
103. Matzinger P. The danger model: a renewed sense of self. *Science* 2002; 296:301-5.
104. Zitvogel L, Galluzzi L, Smyth MJ, Kroemer G. Mechanism of action of conventional and targeted anticancer therapies: reinstating immunosurveillance. *Immunity* 2013; 39:74-88.
105. Galluzzi L, Senovilla L, Zitvogel L, Kroemer G. The secret ally: immunostimulation by anticancer drugs. *Nat Rev Drug Discov* 2012; 11:215-33.
106. Ghiringhelli F, Apetoh L. The interplay between the immune system and chemotherapy: emerging methods for optimizing therapy. *Expert Rev Clin Immunol* 2014; 10:19-30.
107. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* 2006; 313:1960-4.
108. Fridman WH, Pages F, Sautes-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer* 2012; 12:298-306.

109. Senovilla L, Vacchelli E, Galon J, Adjemian S, Eggermont A, Fridman WH, et al. Trial watch: Prognostic and predictive value of the immune infiltrate in cancer. *Oncoimmunology* 2012; 1:1323-43.
110. Galon J, Mlecnik B, Bindea G, Angell HK, Berger A, Lagorce C, et al. Towards the introduction of the 'Immunoscore' in the classification of malignant tumours. *J Pathol* 2014; 232:199-209.
111. Bindea G, Mlecnik B, Tosolini M, Kirilovsky A, Waldner M, Obenauf AC, et al. Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in human cancer. *Immunity* 2013; 39:782-95.
112. Anitei MG, Zeitoun G, Mlecnik B, Marliot F, Haicheur N, Tosi AM, et al. Prognostic and predictive values of the immunoscore in patients with rectal cancer. *Clin Cancer Res* 2014; 20:1891-9.
113. Bindea G, Mlecnik B, Angell HK, Galon J. The immune landscape of human tumors: Implications for cancer immunotherapy. *Oncoimmunology* 2014; 3:e27456.
114. Paroli M, Bellati F, Videtta M, Focaccetti C, Mancone C, Donato T, et al. Discovery of chemotherapy-associated ovarian cancer antigens by interrogating memory T cells. *Int J Cancer* 2014; 134:1823-34.
115. Kohles N, Nagel D, Jungst D, Stieber P, Holdenrieder S. Predictive value of immunogenic cell death biomarkers HMGB1, sRAGE, and DNase in liver cancer patients receiving transarterial chemoembolization therapy. *Tumour Biol* 2012; 33:2401-9.
116. Stoetzer OJ, Wittwer C, Lehner J, Fahmueller YN, Kohles N, Fersching DM, et al. Circulating nucleosomes and biomarkers of immunogenic cell death as predictive and prognostic markers in cancer patients undergoing cytotoxic therapy. *Expert Opin Biol Ther* 2012; 12 Suppl 1:S217-24.
117. Wittwer C, Boeck S, Heinemann V, Haas M, Stieber P, Nagel D, et al. Circulating nucleosomes and immunogenic cell death markers HMGB1, sRAGE and DNase in patients with advanced pancreatic cancer undergoing chemotherapy. *Int J Cancer* 2013; 133:2619-30.
118. Stoll G, Enot D, Mlecnik B, Galon J, Zitvogel L, Kroemer G. Immune-related gene signatures predict the outcome of neoadjuvant chemotherapy. *Oncoimmunology* 2014; 3:e27884.

119. Ghiringhelli F, Apetoh L, Tesniere A, Aymeric L, Ma Y, Ortiz C, et al. Activation of the NLRP3 inflammasome in dendritic cells induces IL-1 β -dependent adaptive immunity against tumors. *Nat Med* 2009; 15:1170-8.
120. Ladoire S, Mignot G, Dabakuyo S, Arnould L, Apetoh L, Rebe C, et al. In situ immune response after neoadjuvant chemotherapy for breast cancer predicts survival. *J Pathol* 2011; 224:389-400.
121. Zitvogel L, Kepp O, Kroemer G. Decoding cell death signals in inflammation and immunity. *Cell* 2010; 140:798-804.
122. Krysko O, Love Aaes T, Bachert C, Vandenabeele P, Krysko DV. Many faces of DAMPs in cancer therapy. *Cell Death Dis* 2013; 4:e631.
123. Garg AD, Dudek AM, Agostinis P. Cancer immunogenicity, danger signals, and DAMPs: what, when, and how? *Biofactors* 2013; 39:355-67.
124. Hou W, Zhang Q, Yan Z, Chen R, Zeh Iii HJ, Kang R, et al. Strange attractors: DAMPs and autophagy link tumor cell death and immunity. *Cell Death Dis* 2013; 4:e966.
125. Garg AD, Martin S, Golab J, Agostinis P. Danger signalling during cancer cell death: origins, plasticity and regulation. *Cell Death Differ* 2014; 21:26-38.
126. Garg AD, Nowis D, Golab J, Vandenabeele P, Krysko DV, Agostinis P. Immunogenic cell death, DAMPs and anticancer therapeutics: an emerging amalgamation. *Biochim Biophys Acta* 2010; 1805:53-71.
127. Honeychurch J, Dive C, Illidge TM. Synchronous apoptosis in established tumors leads to the induction of adaptive immunity. *Oncoimmunology* 2013; 2:e24501.
128. Inoue H, Tani K. Multimodal immunogenic cancer cell death as a consequence of anticancer cytotoxic treatments. *Cell Death Differ* 2014; 21:39-49.
129. Melis MH, Simpson KL, Dovedi SJ, Welman A, MacFarlane M, Dive C, et al. Sustained tumour eradication after induced caspase-3 activation and synchronous tumour apoptosis requires an intact host immune response. *Cell Death Differ* 2013; 20:765-73.
130. Matzinger P. An innate sense of danger. *Ann N Y Acad Sci* 2002; 961:341-2.

131. Lotze MT, Deisseroth A, Rubartelli A. Damage associated molecular pattern molecules. *Clin Immunol* 2007; 124:1-4.
132. Pouwels SD, Heijink IH, ten Hacken NH, Vandenabeele P, Krysko DV, Nawijn MC, et al. DAMPs activating innate and adaptive immune responses in COPD. *Mucosal Immunol* 2014; 7:215-26.
133. Seong SY, Matzinger P. Hydrophobicity: an ancient damage-associated molecular pattern that initiates innate immune responses. *Nat Rev Immunol* 2004; 4:469-78.
134. Obeid M, Panaretakis T, Tesniere A, Joza N, Tufi R, Apetoh L, et al. Leveraging the immune system during chemotherapy: moving calreticulin to the cell surface converts apoptotic death from "silent" to immunogenic. *Cancer Res* 2007; 67:7941-4.
135. Kepp O, Galluzzi L, Martins I, Schlemmer F, Adjemian S, Michaud M, et al. Molecular determinants of immunogenic cell death elicited by anticancer chemotherapy. *Cancer Metastasis Rev* 2011; 30:61-9.
136. Kepp O, Menger L, Vacchelli E, Locher C, Adjemian S, Yamazaki T, et al. Crosstalk between ER stress and immunogenic cell death. *Cytokine Growth Factor Rev* 2013; 24:311-8.
137. Martins I, Tesniere A, Kepp O, Michaud M, Schlemmer F, Senovilla L, et al. Chemotherapy induces ATP release from tumor cells. *Cell Cycle* 2009; 8:3723-8.
138. Martins I, Michaud M, Sukkurwala AQ, Adjemian S, Ma Y, Shen S, et al. Premortem autophagy determines the immunogenicity of chemotherapy-induced cancer cell death. *Autophagy* 2012; 8:413-5.
139. Michaud M, Martins I, Sukkurwala AQ, Adjemian S, Ma Y, Pellegatti P, et al. Autophagy-dependent anticancer immune responses induced by chemotherapeutic agents in mice. *Science* 2011; 334:1573-7.
140. Wang Y, Martins I, Ma Y, Kepp O, Galluzzi L, Kroemer G. Autophagy-dependent ATP release from dying cells via lysosomal exocytosis. *Autophagy* 2013; 9:1624-5.
141. Ma Y, Adjemian S, Yang H, Catani JP, Hannani D, Martins I, et al. ATP-dependent recruitment, survival and differentiation of dendritic cell precursors in the tumor bed after anticancer chemotherapy. *Oncoimmunology* 2013; 2:e24568.

142. Martins I, Wang Y, Michaud M, Ma Y, Sukkurwala AQ, Shen S, et al. Molecular mechanisms of ATP secretion during immunogenic cell death. *Cell Death Differ* 2014; 21:79-91.
143. Lavieri R, Piccioli P, Carta S, Delfino L, Castellani P, Rubartelli A. TLR costimulation causes oxidative stress with unbalance of proinflammatory and anti-inflammatory cytokine production. *J Immunol* 2014; 192:5373-81.
144. Rovere-Querini P, Capobianco A, Scaffidi P, Valentinis B, Catalanotti F, Giazson M, et al. HMGB1 is an endogenous immune adjuvant released by necrotic cells. *EMBO Rep* 2004; 5:825-30.
145. Brusa D, Migliore E, Garetto S, Simone M, Matera L. Immunogenicity of 56 degrees C and UVC-treated prostate cancer is associated with release of HSP70 and HMGB1 from necrotic cells. *Prostate* 2009; 69:1343-52.
146. Arnold T, Michlmayr A, Baumann S, Burghuber C, Pluschnig U, Bartsch R, et al. Plasma HMGB-1 after the initial dose of epirubicin/docetaxel in cancer. *Eur J Clin Invest* 2013; 43:286-91.
147. Candolfi M, Yagiz K, Foulad D, Alzadeh GE, Tesarfreund M, Muhammad AK, et al. Release of HMGB1 in response to proapoptotic glioma killing strategies: efficacy and neurotoxicity. *Clin Cancer Res* 2009; 15:4401-14.
148. Gallucci S, Lolkema M, Matzinger P. Natural adjuvants: endogenous activators of dendritic cells. *Nat Med* 1999; 5:1249-55.
149. Gallucci S, Matzinger P. Danger signals: SOS to the immune system. *Curr Opin Immunol* 2001; 13:114-9.
150. Fredly H, Ersvaer E, Gjertsen BT, Bruserud O. Immunogenic apoptosis in human acute myeloid leukemia (AML): primary human AML cells expose calreticulin and release heat shock protein (HSP) 70 and HSP90 during apoptosis. *Oncol Rep* 2011; 25:1549-56.
151. Aguilera R, Saffie C, Tittarelli A, Gonzalez FE, Ramirez M, Reyes D, et al. Heat-shock induction of tumor-derived danger signals mediates rapid monocyte differentiation into clinically effective dendritic cells. *Clin Cancer Res* 2011; 17:2474-83.

152. Lv LH, Wan YL, Lin Y, Zhang W, Yang M, Li GL, et al. Anticancer drugs cause release of exosomes with heat shock proteins from human hepatocellular carcinoma cells that elicit effective natural killer cell antitumor responses in vitro. *J Biol Chem* 2012; 287:15874-85.
153. Vega VL, Rodriguez-Silva M, Frey T, Gehrmann M, Diaz JC, Steinem C, et al. Hsp70 translocates into the plasma membrane after stress and is released into the extracellular environment in a membrane-associated form that activates macrophages. *J Immunol* 2008; 180:4299-307.
154. Korbelik M, Banath J, Sun J, Canals D, Hannun YA, Separovic D. Ceramide and sphingosine-1-phosphate act as photodynamic therapy-elicited damage-associated molecular patterns: cell surface exposure. *Int Immunopharmacol* 2014; 20:359-65.
155. Galluzzi L, Kepp O, Kroemer G. Mitochondria: master regulators of danger signalling. *Nat Rev Mol Cell Biol* 2012; 13:780-8.
156. Krysko DV, Agostinis P, Krysko O, Garg AD, Bachert C, Lambrecht BN, et al. Emerging role of damage-associated molecular patterns derived from mitochondria in inflammation. *Trends Immunol* 2011; 32:157-64.
157. Zhang Q, Raoof M, Chen Y, Sumi Y, Sursal T, Junger W, et al. Circulating mitochondrial DAMPs cause inflammatory responses to injury. *Nature* 2010; 464:104-7.
158. Martinon F, Petrilli V, Mayor A, Tardivel A, Tschopp J. Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature* 2006; 440:237-41.
159. Shi Y, Mucsi AD, Ng G. Monosodium urate crystals in inflammation and immunity. *Immunol Rev* 2010; 233:203-17.
160. Franchi L, Eigenbrod T, Munoz-Planillo R, Nunez G. The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis. *Nat Immunol* 2009; 10:241-7.
161. Ahrens S, Zelenay S, Sancho D, Hanc P, Kjaer S, Feest C, et al. F-actin is an evolutionarily conserved damage-associated molecular pattern recognized by DNGR-1, a receptor for dead cells. *Immunity* 2012; 36:635-45.

162. Taylor KR, Trowbridge JM, Rudisill JA, Termeer CC, Simon JC, Gallo RL. Hyaluronan fragments stimulate endothelial recognition of injury through TLR4. *J Biol Chem* 2004; 279:17079-84.
163. Scheibner KA, Lutz MA, Boodoo S, Fenton MJ, Powell JD, Horton MR. Hyaluronan fragments act as an endogenous danger signal by engaging TLR2. *J Immunol* 2006; 177:1272-81.
164. Tesniere A, Apetoh L, Ghiringhelli F, Joza N, Panaretakis T, Kepp O, et al. Immunogenic cancer cell death: a key-lock paradigm. *Curr Opin Immunol* 2008; 20:504-11.
165. Sonnemann J, Gressmann S, Becker S, Wittig S, Schmudde M, Beck JF. The histone deacetylase inhibitor vorinostat induces calreticulin exposure in childhood brain tumour cells in vitro. *Cancer Chemother Pharmacol* 2010; 66:611-6.
166. Chao MP, Jaiswal S, Weissman-Tsukamoto R, Alizadeh AA, Gentles AJ, Volkmer J, et al. Calreticulin is the dominant pro-phagocytic signal on multiple human cancers and is counterbalanced by CD47. *Sci Transl Med* 2010; 2:63ra94.
167. Basu S, Binder RJ, Ramalingam T, Srivastava PK. CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin. *Immunity* 2001; 14:303-13.
168. Lauber K, Ernst A, Orth M, Herrmann M, Belka C. Dying cell clearance and its impact on the outcome of tumor radiotherapy. *Front Oncol* 2012; 2:116.
169. Krysko DV, Vandenabeele P. Clearance of dead cells: mechanisms, immune responses and implication in the development of diseases. *Apoptosis* 2010; 15:995-7.
170. Krysko DV, Vanden Berghe T, Parthoens E, D'Herde K, Vandenabeele P. Methods for distinguishing apoptotic from necrotic cells and measuring their clearance. *Methods Enzymol* 2008; 442:307-41.
171. Krysko DV, Vandenabeele P. From regulation of dying cell engulfment to development of anti-cancer therapy. *Cell Death Differ* 2008; 15:29-38.
172. Krysko DV, D'Herde K, Vandenabeele P. Clearance of apoptotic and necrotic cells and its immunological consequences. *Apoptosis* 2006; 11:1709-26.

173. Weiss EM, Frey B, Rodel F, Herrmann M, Schlucker E, Voll RE, et al. Ex vivo- and in vivo-induced dead tumor cells as modulators of antitumor responses. *Ann N Y Acad Sci* 2010; 1209:109-17.
174. Chekeni FB, Elliott MR, Sandilos JK, Walk SF, Kinchen JM, Lazarowski ER, et al. Pannexin 1 channels mediate 'find-me' signal release and membrane permeability during apoptosis. *Nature* 2010; 467:863-7.
175. Ayna G, Krysko DV, Kaczmarek A, Petrovski G, Vandenabeele P, Fesus L. ATP release from dying autophagic cells and their phagocytosis are crucial for inflammasome activation in macrophages. *PLoS One* 2012; 7:e40069.
176. Garg AD, Krysko DV, Vandenabeele P, Agostinis P. The emergence of phox-ER stress induced immunogenic apoptosis. *Oncoimmunology* 2012; 1:786-8.
177. Elliott MR, Chekeni FB, Trampont PC, Lazarowski ER, Kadl A, Walk SF, et al. Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. *Nature* 2009; 461:282-6.
178. Ma Y, Adjemian S, Mattarollo SR, Yamazaki T, Aymeric L, Yang H, et al. Anticancer chemotherapy-induced intratumoral recruitment and differentiation of antigen-presenting cells. *Immunity* 2013; 38:729-41.
179. McDonald B, Pittman K, Menezes GB, Hirota SA, Slaba I, Waterhouse CC, et al. Intravascular danger signals guide neutrophils to sites of sterile inflammation. *Science* 2010; 330:362-6.
180. Aymeric L, Apetoh L, Ghiringhelli F, Tesniere A, Martins I, Kroemer G, et al. Tumor cell death and ATP release prime dendritic cells and efficient anticancer immunity. *Cancer Res* 2010; 70:855-8.
181. Di Virgilio F. Liaisons dangereuses: P2X(7) and the inflammasome. *Trends Pharmacol Sci* 2007; 28:465-72.
182. Riteau N, Gasse P, Fauconnier L, Gombault A, Couegnat M, Fick L, et al. Extracellular ATP is a danger signal activating P2X7 receptor in lung inflammation and fibrosis. *Am J Respir Crit Care Med* 2010; 182:774-83.

183. Cauwels A, Rogge E, Vandendriessche B, Shiva S, Brouckaert P. Extracellular ATP drives systemic inflammation, tissue damage and mortality. *Cell Death Dis* 2014; 5:e1102.
184. Xiang Y, Wang X, Yan C, Gao Q, Li SA, Liu J, et al. Adenosine-5'-triphosphate (ATP) protects mice against bacterial infection by activation of the NLRP3 inflammasome. *PLoS One* 2013; 8:e63759.
185. Gombault A, Baron L, Couillin I. ATP release and purinergic signaling in NLRP3 inflammasome activation. *Front Immunol* 2012; 3:414.
186. Riteau N, Baron L, Villeret B, Guillou N, Savigny F, Ryffel B, et al. ATP release and purinergic signaling: a common pathway for particle-mediated inflammasome activation. *Cell Death Dis* 2012; 3:e403.
187. Iyer SS, Pulsikens WP, Sadler JJ, Butter LM, Teske GJ, Ulland TK, et al. Necrotic cells trigger a sterile inflammatory response through the Nlrp3 inflammasome. *Proc Natl Acad Sci U S A* 2009; 106:20388-93.
188. England H, Summersgill HR, Edye ME, Rothwell NJ, Brough D. Release of Interleukin-1alpha or Interleukin-1beta Depends on Mechanism of Cell Death. *J Biol Chem* 2014; 289:15942-50.
189. Steer SA, Scarim AL, Chambers KT, Corbett JA. Interleukin-1 stimulates beta-cell necrosis and release of the immunological adjuvant HMGB1. *PLoS Med* 2006; 3:e17.
190. Michaud M, Sukkurwala AQ, Martins I, Shen S, Zitvogel L, Kroemer G. Subversion of the chemotherapy-induced anticancer immune response by the ecto-ATPase CD39. *Oncoimmunology* 2012; 1:393-5.
191. Aliagas E, Vidal A, Texido L, Ponce J, Condom E, Martin-Satue M. High expression of ecto-nucleotidases CD39 and CD73 in human endometrial tumors. *Mediators Inflamm* 2014; 2014:509027.
192. Loi S, Pommey S, Haibe-Kains B, Beavis PA, Darcy PK, Smyth MJ, et al. CD73 promotes anthracycline resistance and poor prognosis in triple negative breast cancer. *Proc Natl Acad Sci U S A* 2013; 110:11091-6.
193. Antonioli L, Pacher P, Vizi ES, Hasko G. CD39 and CD73 in immunity and inflammation. *Trends Mol Med* 2013; 19:355-67.

194. Ghiringhelli F, Bruchard M, Chalmin F, Rebe C. Production of adenosine by ectonucleotidases: a key factor in tumor immunoescape. *J Biomed Biotechnol* 2012; 2012:473712.
195. Bastid J, Cottalorda-Regairaz A, Alberici G, Bonnefoy N, Eliaou JF, Bensussan A. ENTPD1/CD39 is a promising therapeutic target in oncology. *Oncogene* 2013; 32:1743-51.
196. Clayton A, Al-Taei S, Webber J, Mason MD, Tabi Z. Cancer exosomes express CD39 and CD73, which suppress T cells through adenosine production. *J Immunol* 2011; 187:676-83.
197. Rao S, Tortola L, Perlot T, Wirnsberger G, Novatchkova M, Nitsch R, et al. A dual role for autophagy in a murine model of lung cancer. *Nat Commun* 2014; 5:3056.
198. Senovilla L, Aranda F, Galluzzi L, Kroemer G. Impact of myeloid cells on the efficacy of anticancer chemotherapy. *Curr Opin Immunol* 2014; 30C:24-31.
199. Ma Y, Galluzzi L, Zitvogel L, Kroemer G. Autophagy and cellular immune responses. *Immunity* 2013; 39:211-27.
200. Li H, Li Y, Jiao J, Hu HM. Alpha-alumina nanoparticles induce efficient autophagy-dependent cross-presentation and potent antitumour response. *Nat Nanotechnol* 2011; 6:645-50.
201. Thorburn J, Horita H, Redzic J, Hansen K, Frankel AE, Thorburn A. Autophagy regulates selective HMGB1 release in tumor cells that are destined to die. *Cell Death Differ* 2009; 16:175-83.
202. Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature* 2002; 418:191-5.
203. Park JS, Gamboni-Robertson F, He Q, Svetkauskaite D, Kim JY, Strassheim D, et al. High mobility group box 1 protein interacts with multiple Toll-like receptors. *Am J Physiol Cell Physiol* 2006; 290:C917-24.
204. Yu M, Wang H, Ding A, Golenbock DT, Latz E, Czura CJ, et al. HMGB1 signals through toll-like receptor (TLR) 4 and TLR2. *Shock* 2006; 26:174-9.
205. Lotze MT, Tracey KJ. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. *Nat Rev Immunol* 2005; 5:331-42.

206. Kokkola R, Andersson A, Mullins G, Ostberg T, Treutiger CJ, Arnold B, et al. RAGE is the major receptor for the proinflammatory activity of HMGB1 in rodent macrophages. *Scand J Immunol* 2005; 61:1-9.
207. Dong Xda E, Ito N, Lotze MT, Demarco RA, Popovic P, Shand SH, et al. High mobility group box I (HMGB1) release from tumor cells after treatment: implications for development of targeted chemoimmunotherapy. *J Immunother* 2007; 30:596-606.
208. Pathak SK, Skold AE, Mohanram V, Persson C, Johansson U, Spetz AL. Activated apoptotic cells induce dendritic cell maturation via engagement of Toll-like receptor 4 (TLR4), dendritic cell-specific intercellular adhesion molecule 3 (ICAM-3)-grabbing nonintegrin (DC-SIGN), and beta2 integrins. *J Biol Chem* 2012; 287:13731-42.
209. Bianchi ME, Manfredi AA. High-mobility group box 1 (HMGB1) protein at the crossroads between innate and adaptive immunity. *Immunol Rev* 2007; 220:35-46.
210. Curtin JF, Liu N, Candolfi M, Xiong W, Assi H, Yagiz K, et al. HMGB1 mediates endogenous TLR2 activation and brain tumor regression. *PLoS Med* 2009; 6:e10.
211. Schiraldi M, Raucci A, Munoz LM, Livoti E, Celona B, Venereau E, et al. HMGB1 promotes recruitment of inflammatory cells to damaged tissues by forming a complex with CXCL12 and signaling via CXCR4. *J Exp Med* 2012; 209:551-63.
212. Tang D, Kang R, Livesey KM, Cheh CW, Farkas A, Loughran P, et al. Endogenous HMGB1 regulates autophagy. *J Cell Biol* 2010; 190:881-92.
213. Kang R, Zeh HJ, Lotze MT, Tang D. The Beclin 1 network regulates autophagy and apoptosis. *Cell Death Differ* 2011; 18:571-80.
214. Kang R, Livesey KM, Zeh HJ, 3rd, Lotze MT, Tang D. HMGB1 as an autophagy sensor in oxidative stress. *Autophagy* 2011; 7:904-6.
215. Yang H, Lundback P, Ottosson L, Erlandsson-Harris H, Venereau E, Bianchi ME, et al. Redox modification of cysteine residues regulates the cytokine activity of high mobility group box-1 (HMGB1). *Mol Med* 2012; 18:250-9.
216. Tang D, Billiar TR, Lotze MT. A Janus tale of two active high mobility group box 1 (HMGB1) redox states. *Mol Med* 2012; 18:1360-2.

217. Venereau E, Casalgrandi M, Schiraldi M, Antoine DJ, Cattaneo A, De Marchis F, et al. Mutually exclusive redox forms of HMGB1 promote cell recruitment or proinflammatory cytokine release. *J Exp Med* 2012; 209:1519-28.
218. Tang D, Kang R, Cheh CW, Livesey KM, Liang X, Schapiro NE, et al. HMGB1 release and redox regulates autophagy and apoptosis in cancer cells. *Oncogene* 2010; 29:5299-310.
219. Kazama H, Ricci JE, Herndon JM, Hoppe G, Green DR, Ferguson TA. Induction of immunological tolerance by apoptotic cells requires caspase-dependent oxidation of high-mobility group box-1 protein. *Immunity* 2008; 29:21-32.
220. Rubartelli A, Lotze MT. Inside, outside, upside down: damage-associated molecular-pattern molecules (DAMPs) and redox. *Trends Immunol* 2007; 28:429-36.
221. Urbonaviciute V, Meister S, Furnrohr BG, Frey B, Guckel E, Schett G, et al. Oxidation of the alarmin high-mobility group box 1 protein (HMGB1) during apoptosis. *Autoimmunity* 2009; 42:305-7.
222. Chiba S, Baghdadi M, Akiba H, Yoshiyama H, Kinoshita I, Dosaka-Akita H, et al. Tumor-infiltrating DCs suppress nucleic acid-mediated innate immune responses through interactions between the receptor TIM-3 and the alarmin HMGB1. *Nat Immunol* 2012; 13:832-42.
223. Tang D, Lotze MT. Tumor immunity times out: TIM-3 and HMGB1. *Nat Immunol* 2012; 13:808-10.
224. Patel J, Bozeman EN, Selvaraj P. Taming dendritic cells with TIM-3: another immunosuppressive strategy used by tumors. *Immunotherapy* 2012; 4:1795-8.
225. Vacchelli E, Galluzzi L, Eggermont A, Fridman WH, Galon J, Sautes-Fridman C, et al. Trial watch: FDA-approved Toll-like receptor agonists for cancer therapy. *Oncoimmunology* 2012; 1:894-907.
226. Vacchelli E, Eggermont A, Sautes-Fridman C, Galon J, Zitvogel L, Kroemer G, et al. Trial Watch: Toll-like receptor agonists for cancer therapy. *Oncoimmunology* 2013; 2:e25238.
227. Yamazaki T, Hannani D, Poirier-Colame V, Ladoire S, Locher C, Sistigu A, et al. Defective immunogenic cell death of HMGB1-deficient tumors: compensatory therapy with TLR4 agonists. *Cell Death Differ* 2014; 21:69-78.

228. Aranda F, Vacchelli E, Obrist F, Eggermont A, Galon J, Sautes-Fridman C, et al. Trial Watch: Toll-like receptor agonists in oncological indications. *Oncoimmunology* 2014; 3:e29179.
229. Apetoh L, Ghiringhelli F, Tesniere A, Criollo A, Ortiz C, Lidereau R, et al. The interaction between HMGB1 and TLR4 dictates the outcome of anticancer chemotherapy and radiotherapy. *Immunol Rev* 2007; 220:47-59.
230. Ma Y, Aymeric L, Locher C, Mattarollo SR, Delahaye NF, Pereira P, et al. Contribution of IL-17-producing gamma delta T cells to the efficacy of anticancer chemotherapy. *J Exp Med* 2011; 208:491-503.
231. Klionsky DJ, Abdalla FC, Abeliovich H, Abraham RT, Acevedo-Arozena A, Adeli K, et al. Guidelines for the use and interpretation of assays for monitoring autophagy. *Autophagy* 2012; 8:445-544.
232. Sukkurwala AQ, Adjemian S, Senovilla L, Michaud M, Spaggiari S, Vacchelli E, et al. Screening of novel immunogenic cell death inducers within the NCI Mechanistic Diversity Set. *Oncoimmunology* 2014; 3:e28473.
233. Vesely MD, Kershaw MH, Schreiber RD, Smyth MJ. Natural innate and adaptive immunity to cancer. *Annu Rev Immunol* 2011; 29:235-71.
234. Zirger JM, Puntel M, Bergeron J, Wibowo M, Moridzadeh R, Bondale N, et al. Immune-mediated loss of transgene expression from virally transduced brain cells is irreversible, mediated by IFN γ , perforin, and TNF α , and due to the elimination of transduced cells. *Mol Ther* 2012; 20:808-19.
235. Tailler M, Senovilla L, Lainey E, Thepot S, Metivier D, Sebert M, et al. Antineoplastic activity of ouabain and pyrithione zinc in acute myeloid leukemia. *Oncogene* 2012; 31:3536-46.
236. Kepp O, Galluzzi L, Lipinski M, Yuan J, Kroemer G. Cell death assays for drug discovery. *Nat Rev Drug Discov* 2011; 10:221-37.
237. Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J Immunol Methods* 1995; 184:39-51.

238. Koopman G, Reutelingsperger CP, Kuijten GA, Keehnen RM, Pals ST, van Oers MH. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood* 1994; 84:1415-20.
239. Galluzzi L, Vitale I, Kepp O, Seror C, Hangen E, Perfettini JL, et al. Methods to dissect mitochondrial membrane permeabilization in the course of apoptosis. *Methods Enzymol* 2008; 442:355-74.
240. Galluzzi L, Zamzami N, de La Motte Rouge T, Lemaire C, Brenner C, Kroemer G. Methods for the assessment of mitochondrial membrane permeabilization in apoptosis. *Apoptosis* 2007; 12:803-13.
241. Zamzami N, Marchetti P, Castedo M, Zanin C, Vayssiere JL, Petit PX, et al. Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death in vivo. *J Exp Med* 1995; 181:1661-72.
242. Mellen MA, de la Rosa EJ, Boya P. Autophagy is not universally required for phosphatidylserine exposure and apoptotic cell engulfment during neural development. *Autophagy* 2009; 5:964-72.
243. Mellen MA, de la Rosa EJ, Boya P. The autophagic machinery is necessary for removal of cell corpses from the developing retinal neuroepithelium. *Cell Death Differ* 2008; 15:1279-90.
244. Segawa K, Kurata S, Yanagihashi Y, Brummelkamp TR, Matsuda F, Nagata S. Caspase-mediated cleavage of phospholipid flippase for apoptotic phosphatidylserine exposure. *Science* 2014; 344:1164-8.
245. Kenis H, Zandbergen HR, Hofstra L, Petrov AD, Dumont EA, Blankenberg FD, et al. Annexin A5 uptake in ischemic myocardium: demonstration of reversible phosphatidylserine externalization and feasibility of radionuclide imaging. *J Nucl Med* 2010; 51:259-67.
246. Galluzzi L, Kepp O, Trojel-Hansen C, Kroemer G. Mitochondrial control of cellular life, stress, and death. *Circ Res* 2012; 111:1198-207.
247. Galluzzi L, Blomgren K, Kroemer G. Mitochondrial membrane permeabilization in neuronal injury. *Nat Rev Neurosci* 2009; 10:481-94.

248. Metivier D, Dallaporta B, Zamzami N, Larochette N, Susin SA, Marzo I, et al. Cytofluorometric detection of mitochondrial alterations in early CD95/Fas/APO-1-triggered apoptosis of Jurkat T lymphoma cells. Comparison of seven mitochondrion-specific fluorochromes. *Immunol Lett* 1998; 61:157-63.
249. Komoriya A, Packard BZ, Brown MJ, Wu ML, Henkart PA. Assessment of caspase activities in intact apoptotic thymocytes using cell-permeable fluorogenic caspase substrates. *J Exp Med* 2000; 191:1819-28.
250. Kepp O, Rajalingam K, Kimmig S, Rudel T. Bak and Bax are non-redundant during infection- and DNA damage-induced apoptosis. *EMBO J* 2007; 26:825-34.
251. Tajeddine N, Galluzzi L, Kepp O, Hangen E, Morselli E, Senovilla L, et al. Hierarchical involvement of Bak, VDAC1 and Bax in cisplatin-induced cell death. *Oncogene* 2008; 27:4221-32.
252. Pauleau AL, Larochette N, Giordanetto F, Scholz SR, Poncet D, Zamzami N, et al. Structure-function analysis of the interaction between Bax and the cytomegalovirus-encoded protein vMIA. *Oncogene* 2007; 26:7067-80.
253. Garg AD, Dudek AM, Agostinis P. Calreticulin surface exposure is abrogated in cells lacking, chaperone-mediated autophagy-essential gene, LAMP2A. *Cell Death Dis* 2013; 4:e826.
254. Hetz C, Chevet E, Harding HP. Targeting the unfolded protein response in disease. *Nat Rev Drug Discov* 2013; 12:703-19.
255. Hetz C, Martinon F, Rodriguez D, Glimcher LH. The unfolded protein response: integrating stress signals through the stress sensor IRE1alpha. *Physiol Rev* 2011; 91:1219-43.
256. Todd DJ, Lee AH, Glimcher LH. The endoplasmic reticulum stress response in immunity and autoimmunity. *Nat Rev Immunol* 2008; 8:663-74.
257. Omasa T, Chen YG, Mantalaris A, Tsai YC, Wu JH. Molecular cloning and sequencing of the human heme-regulated eukaryotic initiation factor 2 alpha (eIF-2 alpha) kinase from bone marrow culture. *DNA Seq* 2002; 13:133-7.
258. Samuel CE. The eIF-2 alpha protein kinases, regulators of translation in eukaryotes from yeasts to humans. *J Biol Chem* 1993; 268:7603-6.

259. Shi Y, Vatter KM, Sood R, An J, Liang J, Stramm L, et al. Identification and characterization of pancreatic eukaryotic initiation factor 2 α -subunit kinase, PEK, involved in translational control. *Mol Cell Biol* 1998; 18:7499-509.
260. Harding HP, Zhang Y, Ron D. Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. *Nature* 1999; 397:271-4.
261. Yang Y, Li XJ, Chen Z, Zhu XX, Wang J, Zhang LB, et al. Wogonin induced calreticulin/annexin A1 exposure dictates the immunogenicity of cancer cells in a PERK/AKT dependent manner. *PLoS One* 2012; 7:e50811.
262. van Schadewijk A, van't Wout EF, Stolk J, Hiemstra PS. A quantitative method for detection of spliced X-box binding protein-1 (XBP1) mRNA as a measure of endoplasmic reticulum (ER) stress. *Cell Stress Chaperones* 2012; 17:275-9.
263. Hayashi A, Kasahara T, Iwamoto K, Ishiwata M, Kametani M, Kakiuchi C, et al. The role of brain-derived neurotrophic factor (BDNF)-induced XBP1 splicing during brain development. *J Biol Chem* 2007; 282:34525-34.
264. Prisci F, Nowak PR, Carrara M, Ali MM. Phosphoregulation of Ire1 RNase splicing activity. *Nat Commun* 2014; 5:3554.
265. Reineke LC, Dougherty JD, Pierre P, Lloyd RE. Large G3BP-induced granules trigger eIF2 α phosphorylation. *Mol Biol Cell* 2012; 23:3499-510.
266. Lundin A, Thore A. Analytical information obtainable by evaluation of the time course of firefly bioluminescence in the assay of ATP. *Anal Biochem* 1975; 66:47-63.
267. McElroy WD, DeLuca MA. Firefly and bacterial luminescence: basic science and applications. *J Appl Biochem* 1983; 5:197-209.
268. Sorensen CE, Novak I. Visualization of ATP release in pancreatic acini in response to cholinergic stimulus. Use of fluorescent probes and confocal microscopy. *J Biol Chem* 2001; 276:32925-32.
269. Imamura H, Nhat KP, Togawa H, Saito K, Iino R, Kato-Yamada Y, et al. Visualization of ATP levels inside single living cells with fluorescence resonance energy transfer-based genetically encoded indicators. *Proc Natl Acad Sci U S A* 2009; 106:15651-6.

270. Tasdemir E, Galluzzi L, Maiuri MC, Criollo A, Vitale I, Hangen E, et al. Methods for assessing autophagy and autophagic cell death. *Methods Mol Biol* 2008; 445:29-76.
271. Klionsky DJ. A human autophagy interaction network. *Autophagy* 2012; 8:439-41.
272. Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, et al. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J* 2000; 19:5720-8.
273. Klionsky DJ. Autophagy in mammalian systems, Part B. Preface. *Methods Enzymol* 2009; 452:xxi-xxii.
274. Mizushima N, Yamamoto A, Hatano M, Kobayashi Y, Kabeya Y, Suzuki K, et al. Dissection of autophagosome formation using Apg5-deficient mouse embryonic stem cells. *J Cell Biol* 2001; 152:657-68.
275. Martins I, Kepp O, Menger L, Michaud M, Adjemian S, Sukkurwala AQ, et al. Fluorescent biosensors for the detection of HMGB1 release. *Methods Mol Biol* 2013; 1004:43-56.
276. Antoine DJ, Harris HE, Andersson U, Tracey KJ, Bianchi ME. A systematic nomenclature for the redox states of high mobility group box (HMGB) proteins. *Mol Med* 2014; 20:135-7.
277. Perne A, Muellner MK, Steinrueck M, Craig-Mueller N, Mayerhofer J, Schwarzingner I, et al. Cardiac glycosides induce cell death in human cells by inhibiting general protein synthesis. *PLoS One* 2009; 4:e8292.
278. Vatakis DN, Koya RC, Nixon CC, Wei L, Kim SG, Avancena P, et al. Antitumor activity from antigen-specific CD8 T cells generated in vivo from genetically engineered human hematopoietic stem cells. *Proc Natl Acad Sci U S A* 2011; 108:E1408-16.
279. Shultz LD, Brehm MA, Garcia-Martinez JV, Greiner DL. Humanized mice for immune system investigation: progress, promise and challenges. *Nat Rev Immunol* 2012; 12:786-98.
280. Payne KJ, Crooks GM. Immune-cell lineage commitment: translation from mice to humans. *Immunity* 2007; 26:674-7.
281. Rongvaux A, Willinger T, Martinek J, Strowig T, Gearty SV, Teichmann LL, et al. Development and function of human innate immune cells in a humanized mouse model. *Nat Biotechnol* 2014; 32:364-72.

282. Ladoire S, Chaba K, Martins I, Sukkurwala AQ, Adjemian S, Michaud M, et al. Immunohistochemical detection of cytoplasmic LC3 puncta in human cancer specimens. *Autophagy* 2012; 8:1175-84.
283. Ladoire S, Hannani D, Vetizou M, Locher C, Aymeric L, Apetoh L, et al. Cell-death-associated molecular patterns as determinants of cancer immunogenicity. *Antioxid Redox Signal* 2014; 20:1098-116.
284. Zitvogel L, Tanchot C, Granier C, Tartour E. Following up tumor-specific regulatory T cells in cancer patients. *Oncoimmunology* 2013; 2:e25444.
285. Popadic D, Anegón I, Baeten D, Eibel H, Giese T, Marits P, et al. Predictive immunomonitoring -- the COST ENTIRE initiative. *Clin Immunol* 2013; 147:23-6.
286. Jager M, Schoberth A, Ruf P, Hess J, Hennig M, Schmalfeldt B, et al. Immunomonitoring results of a phase II/III study of malignant ascites patients treated with the trifunctional antibody catumaxomab (anti-EpCAM x anti-CD3). *Cancer Res* 2012; 72:24-32.
287. Rainov NG. A phase III clinical evaluation of herpes simplex virus type 1 thymidine kinase and ganciclovir gene therapy as an adjuvant to surgical resection and radiation in adults with previously untreated glioblastoma multiforme. *Hum Gene Ther* 2000; 11:2389-401.
288. Fillat C, Carrio M, Cascante A, Sangro B. Suicide gene therapy mediated by the Herpes Simplex virus thymidine kinase gene/Ganciclovir system: fifteen years of application. *Curr Gene Ther* 2003; 3:13-26.

Legends to Figures

Figure 1. Molecular and cellular mechanisms of immunogenic cell death. Cancer cells succumb to specific stimuli (e.g., anthracyclines, oxaliplatin, some forms of radiation therapy, photodynamic therapy) while emitting a spatiotemporally ordered combination of damage-associated molecular patterns (DAMPs). These signals include (but are not limited to) the pre-apoptotic exposure of the endoplasmic reticulum chaperone calreticulin (CALR) on the surface of dying cells, the secretion of ATP during the blebbing phase of apoptosis, and the release of the nuclear protein high mobility group box 1 (HMGB1) upon plasma membrane permeabilization. Upon binding to specific receptors, immunogenic cell death (ICD)-associated DAMPs promote the recruitment of antigen-presenting cells (APCs) and stimulate their ability to take up particulate material and cross-present exogenous antigens to CD8⁺ cytotoxic T lymphocytes (CTLs) while secreting interleukin (IL)-1 β . The consequent adaptive immune response also involves $\gamma\delta$ T lymphocytes that produce IL-17. Both $\gamma\delta$ T cells and $\alpha\beta$ CTLs mediate direct antineoplastic effects by secreting interferon γ (IFN γ) and via the granzyme-perforin pathway. In addition, some CTLs acquire a memory phenotype, underlying the establishment of long-term immunological protection.

Figure 2. Assays for the evaluation of immunogenic cell death *in vivo*. A. Vaccination assays. Murine cancer cells of choice are exposed *in vitro* to a putative inducer of immunogenic cell death (ICD), 1 μ M mitoxantrone (positive control) or 50 μ M cisplatin (negative control) for a predetermined time (normally 6-24 hours), then washed, resuspended in PBS, and eventually injected s.c. into one flank (vaccination site) of immunocompetent syngeneic mice (ideally 5-10 per group). One week later, mice are challenged with living cancer cells of the same type, which are inoculated s.c. into the contralateral flank (challenge site). Tumor incidence and growth are routinely monitored at both injection sites over a 1-2 months period. The development of neoplastic lesions at the vaccination site indicates that the stimulus under investigation is unable to cause cell death (under the circumstances under investigation) to a degree that is compatible with the

elicitation of adaptive immunity. Conversely, in the absence of tumors at the vaccination site, the ability of the experimental maneuver under evaluation to promote *bona fide* ICD inversely correlates with the number of neoplastic lesions developed at the challenge site. As an indication, neoplastic cells exposed *in vitro* to 1 μ M mitoxantrone for 6 hours and maintained in culture for additional 18 hours vaccinate approximately 80% of mice against a challenge with living cells of the same type. **B. Therapeutic assays.** Immunocompetent and immunodeficient syngeneic mice bearing grafted, genetically-driven or chemically-induced subcutaneous or orthotopic tumors are treated with a putative ICD inducer, mitoxantrone (positive control) or cisplatin (negative control) at therapeutic doses, followed by the monitoring of tumor size over a 1-3 weeks period. In this setting, *bona fide* ICD inducers mediate optimal antineoplastic effects in immunocompetent, but not in immunodeficient, mice. Since this is also the case of therapeutic interventions that exert off-target immunostimulatory effects, this assay cannot be employed alone to discriminate between ICD and non-immunogenic cell death (nICD). Please note that all curves represented in this figure do not depict primary data but have been created for the sake of exemplification.

Table 1. Assays for the detection of immunogenic cell death-associated processes *in vitro*.

Process	Parameter	Platform	Main advantage	Main disadvantage	Notes
Cell death	BAX activation	Flow cytometry Fluorescence microscopy Immunoblotting	Compatible with real-time detection	Real-time detection requires transgenic cell lines	Based on conformation-specific antibodies or cell lines expressing GFP-tagged BAX
	$\Delta\psi_m$ dissipation	Flow cytometry Fluorescence microscopy Fluorometry	Early process in the cascade of events leading to cell death	The $\Delta\psi_m$ can be dissipated in the course of cell death-unrelated processes	Several $\Delta\psi_m$ -sensitive probes with different spectral and biochemical properties are available, including DiOC(3) and CMTMRos
	Caspase activation	Flow cytometry IF microscopy Fluorescence microscopy Fluorometry Immunoblotting	Directly involved in CALR exposure	Some caspases get activated in the course of cell death-unrelated processes	Antibodies specific for active caspases or their substrates, as well as self-quenched peptides that emit upon cleavage are available
	Nuclear pyknosis	Fluorescence microscopy	Compatible with simultaneous assessments	Prone to underestimation, owing to the detachment of cells from the substrate	Based on chromatinophilic dyes like Hoechst 33342 or cell lines expressing RFP-tagged variants of H2B
	PMP	Flow cytometry Fluorescence microscopy Light microscopy	Straightforward and very reliable indicator of cell death	End-stage measurement	Several exclusion dyes with different spectral properties are available, including trypan blue, DAPI and PI
	Surface-exposed PS	Flow cytometry Fluorescence microscopy	Compatible with simultaneous assessments	PS exposure does not always accompany cell death	Based on fluorochrome-tagged variants of the protein annexin A5
CALR exposure	Surface-exposed CALR	Flow cytometry Fluorescence microscopy Native gels	Compatible with real-time detection and simultaneous assessments	Real-time detection requires transgenic cell lines	Based on CALR-specific antibodies, cell lines expressing HaloTag TM -tagged CALR variants, GFP-tagged CALR variants, or GFP-tagged PDIA3 variants, or the quantification of cell surface proteins upon biotinylation
ER stress	Phosphorylation of EIF2A or EIF2A kinases	IF microscopy Immunoblotting	EIF2A phosphorylation is required in CALR exposure	Incompatible with high-throughput platforms	Based on phosphoepitope-specific antibodies
	<i>XBP1</i> splicing	Fluorescence microscopy	Compatible with real-time detection	Incomplete assessment of the ER stress response	Based on cell lines expressing a fluorescent variant of XBP1

	ATF6 activation	Fluorescence microscopy	Compatible with real-time detection	Incomplete assessment of the ER stress response	Based on cell lines expressing a fluorescent variant of ATF6
	Formation of stress granules	Fluorescence microscopy	Compatible with real-time detection	Not specific for ER stress	Based on cell lines stably expressing a GFP-tagged variant of G3BP1
ATP secretion	Extracellular ATP	Luminometry HPLC-MS	Very sensitive and compatible with real-time detection	Extracellular ATP is exposed to several ectonucleotidases	Extracellular ATP can be monitored in culture supernatants or in cells stably expressing luciferase on their surface
	Cytosolic ATP	Fluorescence microscopy Luminometry HPLC-MS	Cytosolic ATP is more stable than its extracellular counterpart	Indirect indication of ATP secretion	Residual cytosolic ATP can be monitored upon cell lysis or in cells expressing ATP-sensitive FRET-based probes
	Vesicular ATP	Flow cytometry Fluorescence microscopy	Compatible with real-time detection	Indirect indication of ATP secretion	Based on the fluorescent probe quinacrine
Autophagy	Autophagosome formation	Fluorescence microscopy Immunoblotting Other techniques	Can be monitored with a large panel of techniques	Autophagy is not always required for the secretion of ATP in the course of ICD	Cell lines stably expressing GFP-LC3 offer a means to monitor the formation of autophagic vacuoles in real-time
HMGB1 release	Extracellular HMGB1	ELISA Immunoblotting Mass spectroscopy	Very sensitive and compatible with real-time detection	Relatively expensive	ELISA kits for the detection of HMGB1 are available from commercial providers
	Intracellular HMGB1	Fluorescence microscopy Immunoblotting	Compatible with real-time detection	Indirect indication of HMGB1 release	Based on HGMB1-specific antibodies or cell lines expressing fluorescent variants of HMGB1

Abbreviations: ATF6, activating transcription factor 6; BAX, BCL2-associated X protein; CALR, calreticulin; CMTMRos, chloromethyltetramethylrosamine; $\Delta\psi_m$, mitochondrial transmembrane potential; DAPI, 4',6-diamidino-2-phenylindole; DiOC(3), 3,3'-dihexyloxacarbocyanine iodide; EIF2A, eukaryotic translation initiation factor 2A; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; FRET, fluorescence resonance energy transfer; G3BP1, GTPase activating protein (SH3 domain) binding protein 1; GFP, green fluorescence protein; H2B, histone 2B; HGMB1, high mobility group box 1; HPLC, high-performance liquid chromatography; ICD, immunogenic cell death; IF, immunofluorescence; MS, mass spectrometry; PDIA3, protein disulfide isomerase family A, member 3; PI, propidium iodide; PMP, plasma membrane permeabilization; PS, phosphatidylserine; RFP, red fluorescent protein; XBP1, X-box binding protein 1.

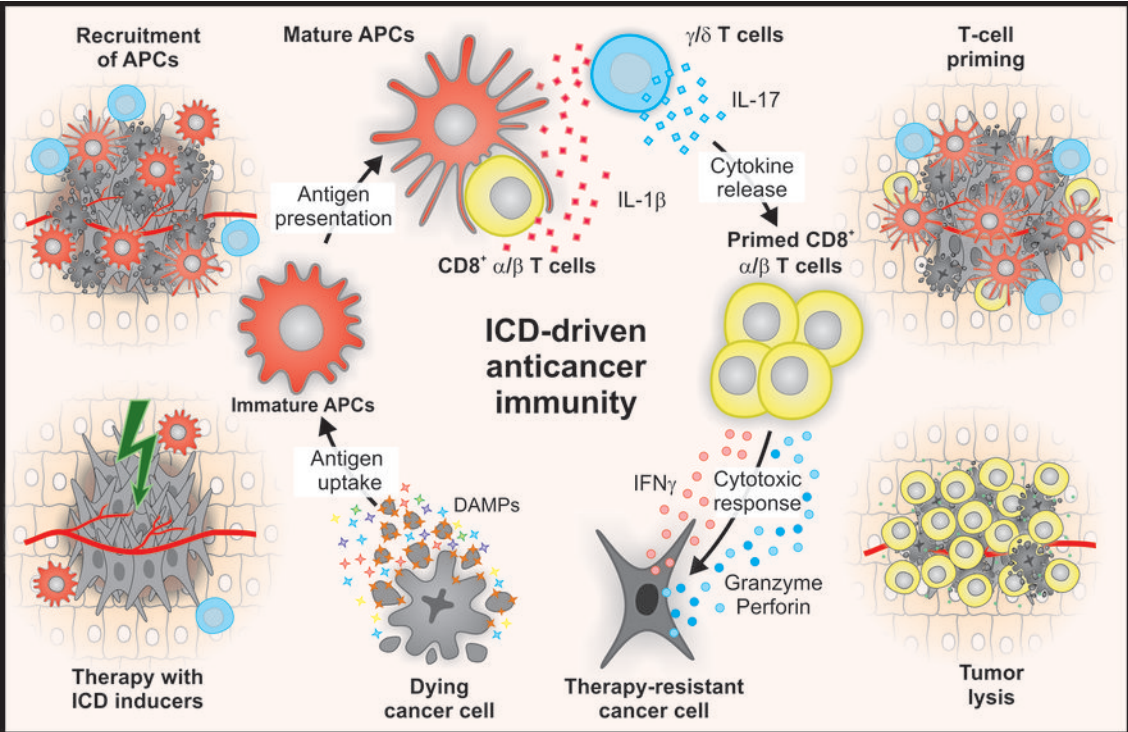


Figure 1

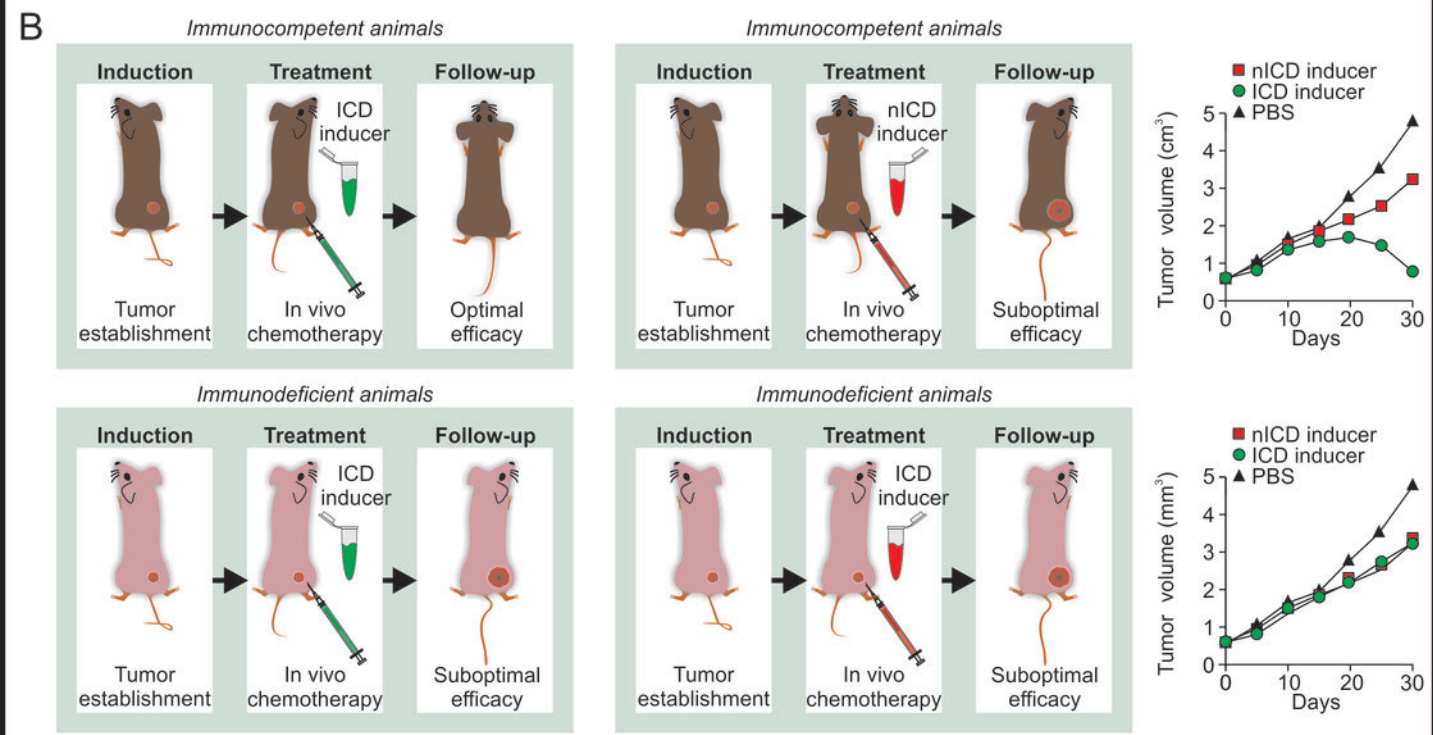
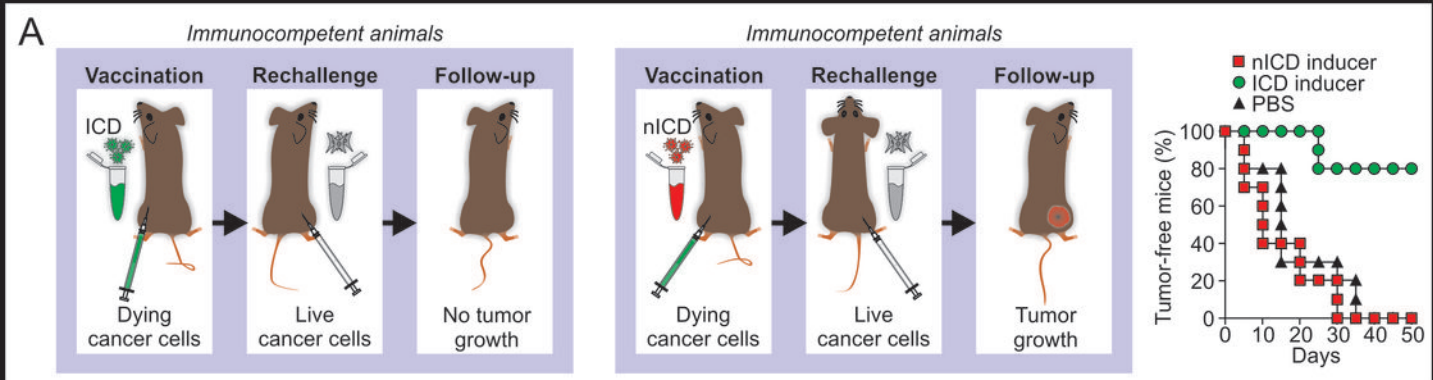


Figure 2